CHARACTERIZATION OF A FUNCTIONAL ROLE OF THE NEUROKININ-3 RECEPTOR IN BEHAVIORAL EFFECTS OF COCAINE

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ABSTRACT

CHARACTERIZATION OF A FUNCTIONAL ROLE OF THE NK-3 RECEPTOR IN BEHAVIORAL EFFECTS OF COCAINE

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The tachykinin NK-3 receptor is a G-protein coupled receptor activated by mammalian tachykinin neuropeptides, which can modulate dopaminergic neurotransmission, and alter dopamine-mediated behaviors. The NK-3 receptor is currently under investigation as a novel therapeutic target for cocaine addiction. Our studies, as outlined in this dissertation, sought to determine if NK-3 receptors have a functional role in the acute as well as long-term behavioral effects of cocaine.

Administration of NK-3 receptor agonists or antagonists potentiates or attenuates dopamine-mediated behaviors, respectively. Based on these findings, we hypothesized that blockade of neurokinin-3 receptors would alter acute and long-term behavioral responses to cocaine. We investigated whether acute and repeated administration of the NK-3 receptor antagonist SB 222200 altered hyperactivity induced by cocaine, and determined a possible mechanism involving dopamine D1 receptors in the striatum. We also determined whether NK-3 receptor blockade altered the development and expression of behavioral sensitization after repeated cocaine administration. Lastly, we investigated whether modulation of behavioral effects of acute and repeated cocaine by NK-3 receptors involved GSK3 phosphorylation in the nucleus accumbens.

As described in this dissertation, we show that acute administration of the NK-3 receptor antagonist SB 222200 before a cocaine injection attenuated stereotypic responses produced by cocaine. Repeated administration of SB 222200 enhanced stereotypic activity produced by either cocaine or a low dose of SKF 82958 (0.125 mg/kg, i.p.) when administered seven days later. Dopamine receptor binding studies were performed to determine the mechanism of enhanced stereotypic responses. Binding studies showed a 19.7% increase in dopamine D1 receptor density in the striatum seven days later after repeated SB 222200 administration. These findings demonstrate that acute blockade of NK-3 receptors attenuated cocaine-induced behaviors in agreement with previous studies. Furthermore, these studies also show novel effects of repeated blockade of NK-3 receptors, which causes subsequent enhancement of cocaine and dopamine D1 receptor-mediated behaviors, possibly resulting from dopamine D1 receptor up-regulation in the striatum.

In order to determine a role of NK-3 receptors in the development of cocaine-induced behavioral sensitization, the NK-3 receptor antagonist SB 222200 (2.5 or 5 mg/kg, s.c.) was administered prior to daily cocaine injections for 5 days. After a 7-day drug-free period, behavioral responses to a cocaine challenge were measured. Repeated administration of cocaine for 5 days induced a sensitized response upon a cocaine challenge 7 days later. Administration of SB 222200 prior to daily cocaine attenuated the development of behavioral sensitization. Moreover, administration of SB 222200 prior to the cocaine challenge blocked the expression of behavioral sensitization. These findings demonstrate that NK-3 receptor activity is involved in the development and expression of behavioral sensitization to cocaine.

Lastly, we examined GSK3 phosphorylation in the nucleus accumbens induced by acute and repeated cocaine administration and determined if phosphorylation was altered by NK-3 receptor blockade. Similar to the drug administration regimens used in the behavioral studies, the NK-3 receptor antagonist SB 222200 was administered 30 mins prior to an acute cocaine injection. The nucleus accumbens was examined for changes in GSK3 phosphorylation by Western blot analysis. Increases in phosphorylation of the isoforms, GSK3α and GSK3β in the nucleus accumbens were detected 20 mins after an acute injection of cocaine. NK-3 receptor blockade prior to cocaine administration did not alter the cocaine-induced increase in GSK3 phosphorylation.

Similar to the behavioral sensitization studies, SB 222200 was administered prior to repeated cocaine for 5 days, and 7 days later GSK3 phosphorylation was measured after a subsequent cocaine challenge. In contrast to the increases in GSK3α and GSK3β in the nucleus accumbens after an acute cocaine injection, no regulation of GSK3 phosphorylation was found after prior repeated cocaine administration and cocaine challenge. Administration of SB 222200 prior to repeated cocaine produced an increase in GSK3α and GSK3β phosphorylation after a cocaine challenge. Collectively, these data point to involvement of NK-3 receptor activity in changes in the phosphorylation of GSK3 in the nucleus accumbens produced by cocaine.

In summary, functional involvement of NK-3 receptors in acute and long-term behavioral effects of cocaine was investigated. In agreement with previous findings, studies in this dissertation demonstrate that acute blockade of NK-3 receptors attenuates cocaine-induced behaviors. In addition, we found novel effects of repeated blockade of NK-3 receptors on cocaine-induced hyperactivity. There is enhancement of subsequent cocaine and dopamine D1 receptor-mediated behaviors possibly due to dopamine D1 receptor up-

regulation in the striatum. NK-3 receptor activity was shown to be involved in long-term behavioral effects of cocaine and molecular changes in GSK3 phosphorylation in the nucleus accumbens. Blockade of NK-3 receptors prevented the development and expression of behavioral sensitization to cocaine and also blocked the changes in the phosphorylation of GSK3 in the nucleus accumbens. This dissertation has demonstrated a role of NK-3 receptors in modulating acute as well long-term cocaine-induced behavioral hyperactivity. Therefore, there is potential clinical relevance of NK-3 receptors in cocaine abuse and dependence as a therapeutic target for treatment, which warrants further characterization in future preclinical and clinical investigations.

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DEDICATION

In memory of my grandmothers, wonderful mothers to my parents, whose generosity, patience and kind-heartedness will always inspire me.

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6OHDA	6-hydroxydopamine
Akt	protein kinase B
ANOVA	analysis of variance
CNS	central nervous system
cAMP	cyclic adenosine 5' monophosphate
CREB	cAMP-responsive element binding protein
DAG	diacylglycerol
DARPP-32	dopamine- and cAMP-regulated phosphoprotein, M(r) 32kDa
DAT	dopamine transporter
DOPAC	3,4-dihydroxyphenylacetic acid
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders
FDA	Food and Drug Administration
GPCR	G-protein coupled receptor
GSK3	glycogen synthase kinase-3
GSK3α	glycogen synthase kinase-3 alpha
GSK3β	glycogen synthase kinase-3 beta
IP	intraperitoneal
IP3	1,4,5 inositol triphosphate
MAPKAP-1	MAP-kinase activated protein kinase-1
NET	norepinephrine transporter
NK-3	tachykinin receptor
NK-A	neurokinin-A
NK-B	neurokinin-B
NMDA	N-methyl-D-aspartic acid
NP-γ	neuropeptide-gamma
NP-K	neuropeptide-K
PIP2	phosphoinositol 4,5 biphosphate
PLC	phospholipase C
PPTA	preprotachykinin-A
PPTB	preprotachykinin-B
SERT	serotonin transporter
STEP	striatal-enriched tyrosine phosphatase
TACR3	NK-3 gene
TDZD	4-benzyl-2-methyl-1,2,4,-thiadiazolidine-3,5-dione
VTA	ventral tegmental area

CHAPTER 1

GENERAL INTRODUCTION

Scientific rationale

Presently, 30 million Americans over the age of 12 reportedly have used cocaine, and 2.4 million used cocaine within the past month, according to recent reports from the National Survey on Drug Use and Health. Cocaine has toxic effects that damages the cerebrovascular, cardiovascular, hepatic, pulmonary, genitourinary, and gastrointestinal systems (Conway and Tamargo, 2001; Fessler *et al*, 1997; Glauser and Queen, 2007; Mendelson and Mello, 1996; Pozner *et al*, 2005; van den Brink and van Ree, 2003). Cocaine use can also transition into compulsive drug-taking wherein individuals would be diagnosed with either cocaine abuse or dependence based on DSM-IV criteria. There are no FDA-approved pharmacotherapies for the treatment of cocaine addiction, which has prompted extensive studies in search of novel therapeutic targets. One of the novel targets currently being studied is the tachykinin NK-3 receptor (Silva *et al*, 2008). The general objectives of the studies conducted in this dissertation were to examine effects of NK-3 receptor blockade on behavioral effects of cocaine, and to determine possible underlying mechanisms. The overall intent of this research was to further characterize the NK-3 receptor as a potential therapeutic target in the treatment of cocaine addiction.

Anatomical and functional evidence point to a role of NK-3 receptors in regulating dopaminergic neurotransmission. NK-3 receptor activity modulates dopamine-mediated behaviors (Bishop and Walker, 2004; de Souza Silva *et al*, 2006a, b; Jocham *et al*, 2007; Jocham *et al*, 2006) by altering dopaminergic neuronal activity in the substantia nigra and ventral tegmental area (Keegan *et al*, 1992; Overton *et al*, 1992) and dopamine

outflow and metabolism in the striatum, nucleus accumbens and prefrontal cortex (Bannon *et al*, 1995; Humpel *et al*, 1991; Marco *et al*, 1998). Since evidence indicates that NK-3 receptors regulate dopaminergic neurotransmission and behaviors, we hypothesized that blockade of NK-3 receptors would alter acute and long-term behavioral responses to cocaine. The following specific aims were devised to address this hypothesis.

1. To examine effects of acute and repeated NK-3 receptor blockade on behavioral responses to cocaine.
2. To determine consequences of NK-3 receptor blockade on the development and expression of behavioral sensitization to cocaine.
3. To elucidate possible involvement of NK-3 receptors and GSK3 phosphorylation in the nucleus accumbens.

Studies conducted in this dissertation investigated acute and long-term effects of NK-3 receptor blockade on cocaine-induced hyperactivity, revealing a role of dopamine D1 receptors in the striatum. A functional role of NK-3 receptors in behavioral sensitization to cocaine was also investigated and possible mechanisms involving GSK3 phosphorylation in the nucleus accumbens were explored.

Clinical aspects of cocaine abuse and addiction

Recent estimates from the 2006 National Survey on Drug Use and Health (NSDUH) show that 35.3 million Americans over the age of 12 have used cocaine. A 2.4 million reportedly used cocaine over the past month, and there is an estimated 977,000 new users yearly over the age of 18. Cocaine can be taken either orally, intranasally, intravenously,

inhaled or smoked, and is usually taken in repeated binges. Cocaine is used illicitly either in the forms of coca paste, cocaine powder or the lipid soluble crack cocaine. Use of cocaine brings about subjective effects described as intense euphoria and alertness, increased confidence and strength, heightened sexual feeling and a care-free state (Mendelson *et al*, 1996). There is usually concurrent abuse of other drugs in addition to cocaine such as benzodiazepines, opioids, and alcohol (van den Brink *et al*, 2003). The diverse forms of cocaine used, routes of administration and concurrent abuse of other drugs potentially complicate the diagnosis of degree of dependence and abuse, and may confound determination of the efficacy of medications and regimens used in treatment of cocaine addiction (Mendelson *et al*, 1996).

Cocaine abuse and dependence are a part of a spectrum of substance-abuse disorders that have been classified based on DSM-IV criteria (Mendelson *et al*, 1996). Cocaine abuse is defined as a pattern of continued substance use that impairs work, school, home and social living, despite having persistent social and legal problems, and use in dangerous situations. Cocaine dependence is diagnosed based on evidence of drug tolerance, compulsive drug acquisition and withdrawal in addition to criteria for cocaine abuse. Following cessation of heavy and prolonged cocaine use, the DSM-IV criteria for withdrawal consists of depressed mood and two of the following symptoms that include fatigue, vivid and unpleasant dreams, insomnia or hypersomnia, increased appetite, and psychomotor retardation or agitation. There are other psychiatric and affective disorders associated with cocaine use such as disorders in sexual function, sleep, anxiety, mood, delirium, panic disorders and paranoid psychosis (Mendelson *et al*, 1996; van den Brink *et al*, 2003).

In addition to its potential for abuse and to cause drug dependence, cocaine produces toxic insults to all systems in the body. Cocaine use may predispose individuals to have subarachnoid hemorrhage due to ruptured intracerebral aneurysms that can result in death. Cocaine can also cause cerebral vasospasm, seizures and ischemic stroke (Conway *et al*, 2001; Fessler *et al*, 1997; Glauser *et al*, 2007). Cocaine-induced toxicity can include acute cardiovascular effects such as acute myocardial infarction, barotrauma and dysrhythmias, and also chronic effects such as acceleration of hypertensive disease, coronary atherosclerosis, left ventricular hypertrophy, myocarditis and endocarditis (Pozner *et al*, 2005). Cocaine can cause acute renal failure and renal infarction, acute tubular necrosis, and bowel ischemia. Chronic use of cocaine by inhalation can cause perforation and necrosis of the nasal septum and also acute pulmonary syndromes such as crack lung, bronchiolitis obliterans with organizing pneumonia, pulmonary hemorrhage with eosinophilia, noncardiogenic pulmonary edema, interstitial pulmonary disease and exacerbation of asthmatic attacks. Cocaine use can cause obstetrical complications such as placental abruption, abnormal labor, spontaneous abortion, premature rupture of membranes and uterine rupture, as well as cause post-natal complications (Glauser *et al*, 2007).

Currently, treatment programs for cocaine abusers have a low retention rate, and abusers seeking treatment have a high relapse rate (van den Brink *et al*, 2003). Predictors of poor treatment outcome include presence of poly drug use, ongoing alcohol use, smoking, recent and more frequent cocaine use with a positive cocaine urine test during the time of admission to a treatment program, and high severity of cocaine withdrawal prior to entering a treatment program. Poor treatment retention is also frequent among

patients with impulsive and novelty seeking personality traits (Poling *et al*, 2007). Predictors of relapse to cocaine abuse include co-existing depression that increases risk of relapse, high stress response in measures of cortisol, epinephrine or norepinephrine, impaired cognitive performance in response to psychological stress, and heightened cue-induced activation of brain regions such as the sensory association cortex, motor cortex, and posterior cingulate cortex (Poling *et al*, 2007). In addition to identifying patients at risk for relapse and poor treatment outcome, information of recurrent drug use, concurrent alcohol abuse, extent of cocaine withdrawal and presence of co-morbid depression may be useful in monitoring of treatment progression in patients (Poling *et al*, 2007).

There are a number of medications used to treat cocaine-related psychological disorders, however there are no effective pharmacotherapies at present to treat addiction. Antidepressant drugs such as desipramine are used to treat cocaine withdrawal-related dysphoria, and propanolol is used to treat severe cocaine withdrawal (Mendelson *et al*, 1996; van den Brink *et al*, 2003). Drug classes that have been tried in treatment of addiction in patients include drugs acting on dopaminergic transmission such as the dopamine D2/D3 receptor agonist bromocriptine and the dopamine transporter inhibitor, mazindol. Opioid drugs such as buprenorphine, and anticonvulsant drugs such as gabapentin have also been used (Mendelson *et al*, 1996). A number of factors may play into lack of effectiveness of these medications, however some medications can be effective in certain type of patients. For instance, patients with high severity of cocaine withdrawal show better treatment responses to propanolol, but gabapentin is more effective with low severity withdrawal (Poling *et al*, 2007). Behavioral modification

therapies can be used concurrently with medications and include aversion therapy, network therapy, behavioral treatment, contingency-based contracts and cognitive therapy (Mendelson *et al*, 1996) in the management of cocaine addiction.

In summary, there are a number of obstacles in the current management and treatment of cocaine addiction. The various routes of administration and presence of poly-drug abuse may complicate the efficacy of medications, and effective treatment regimens may vary amongst individuals. Poor treatment outcome and relapse is predicted by factors such as the current poly-drug use, high severity of cocaine withdrawal and recurrent drug use prior to treatment entry, and co-morbid depression. Pharmacotherapies with behavioral modification and also monitoring of treatment progression may be beneficial in the management of cocaine addiction.

Molecular characterization of the NK-3 receptor

The NK-3 receptor is one of three mammalian tachykinin receptors that also include the NK-1 and NK-2 receptors. The tachykinin receptors possess key structural features shared by members of the G- protein coupled receptor (GPCR) super-family. The amino acid sequence of the NK-3 receptor has seven hydrophobic segments (Buell *et al*, 1992; Shigemoto *et al*, 1990), which corresponds to the seven trans-membrane domains of GPCRs. There are four potential N-glycosylation sites at the amino terminus and also two serine/threonine phosphorylation sites at the third cytoplasmic loop and 28 at the carboxyl terminus, which may play a role in the propensity of the receptor to desensitize (Shigemoto *et al*, 1990). Conserved cysteine residues located in the first and second extracellular loops of the NK-3 receptor are thought to form a disulfide bond, and another conserved cysteine residue located at the seventh transmembrane domain serves

to anchor the receptor to the plasma membrane through palmitoylation. Conserved proline and glycine residues in the transmembrane domains aid in locking transmembrane domain helical structure of the receptor by forming bends. Interactions between the fifth and seventh transmembrane domains are also important for receptor structural stability, as demonstrated by decreased agonist and antagonist binding in chimeric receptor constructs that have incompatible fifth and seventh transmembrane domains (Gether *et al*, 1993). Overall, these conserved motifs stabilize the structure of the NK-3 receptor.

Distinct structural features of the NK-3 receptor determine functional interaction with the endogenous tachykinins. In particular, the seventh transmembrane domain and carboxyl terminus of the NK-3 receptor are important to the interaction with the carboxyl terminus common to all tachykinins (Gether *et al*, 1993; Shigemoto *et al*, 1990). However, ligand-specific interactions are determined by other regions of the NK-3 receptor. Ligand binding studies with chimeric receptor constructs show that epitopes within the amino terminus and/or the first transmembrane domain are important for recognition of substance P (Gether *et al*, 1993) and those in the carboxyl terminus are important for neurokinin B (NK-B). Other recognition sites also determine ligand selectivity for NK-3 receptor over other tachykinins receptors. For instance, NK-B binding to the NK-3 receptor is 69-fold more selective than neurokinin A (NK-A) and 452-fold more than substance P (Shigemoto *et al*, 1990).

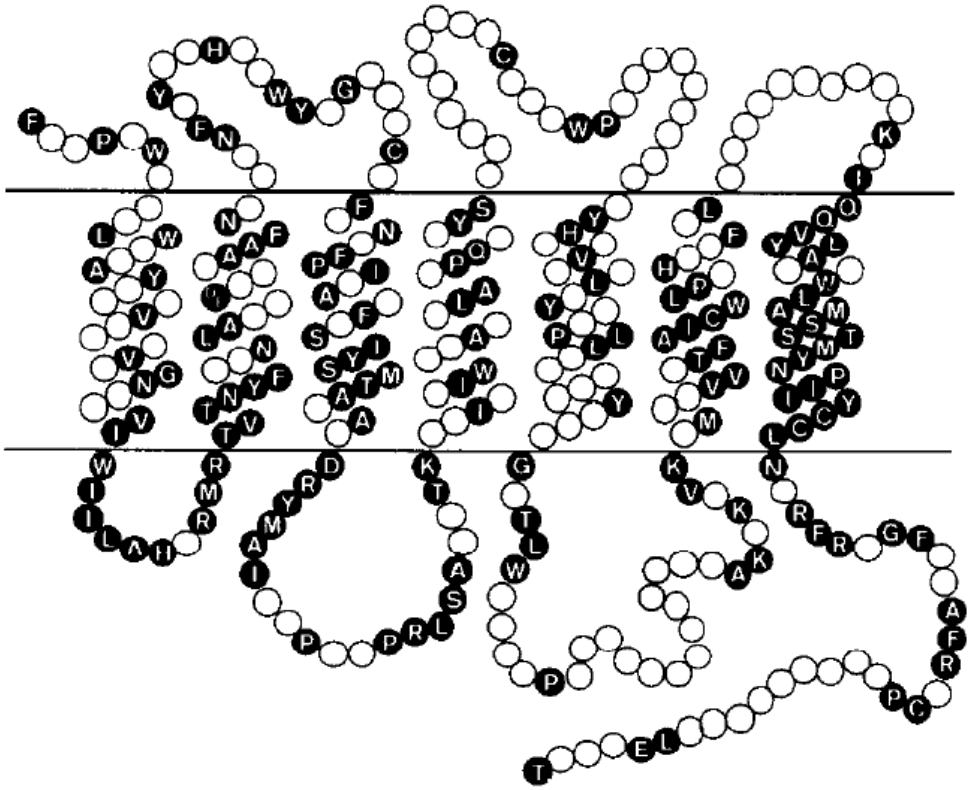

Figure 1.1: A seven transmembrane structure common to tachykinin receptors from molecular studies in rat. Conserved amino acid residues in the three mammalian tachykinin receptors are illustrated in black circles and divergent residues are in white circles (Adapted from Shigemoto *et al*, 1990).

In addition, NK-B is 240-fold more selective for NK-3 over NK-1 receptors, but substance P is 2000-fold more for NK-1 over NK-3 receptors (Gether *et al*, 1993). Overall, these binding studies suggest that different regions of the NK-3 receptor are involved in recognition and selectivity of the endogenous ligands NK-B and substance P.

The three mammalian tachykinin receptors share similarities in their amino acid sequences and in their structure as illustrated in Figure 1.1, with the rat NK-3 receptor having 66.3% homology with the NK-1 receptor and 54.9% with NK-2 receptor (Shigemoto *et al*, 1990). Despite their similarities, the tachykinin receptors have different structural and functional features. They differ in the length of their amino acid sequences, with the NK-3 receptor being the largest of the three, 452 amino acids long, the NK-1 receptor having 407, and the NK-2 receptor 384 (Maggi, 1995). The tachykinin receptors also diverge in the sequences of both their amino and carboxyl terminus (Shigemoto *et al*, 1990), which may play a role in ligand recognition and signal transduction, respectively. For instance, as compared to the four putative N-glycosylation sites in the NK-3 receptor, there are two in the NK-1 receptor and one in the NK-2 receptor. There are also differences in the number of serine/threonine residues in the third cytoplasmic loop and in the carboxyl terminus among the tachykinin receptors; the NK-3 receptor having two in the third cytoplasmic loop and 28 in the carboxyl terminus, NK-1 receptor having five and 26 and NK-2 receptor having one and 14. These features may play a role in the receptor's propensity for desensitization. Compared to other tachykinin receptors, the NK-1 receptor rapidly desensitize upon continued agonist application, the NK-3 receptor moderately desensitizes, and the NK-2 receptor weakly desensitizes (Garland *et al*, 1996; Maggi, 1995; Ohkubo and Nakanishi, 1991; Raddatz *et al*, 1995; Shigemoto *et al*, 1990).

The main signal transduction pathway activated by NK-3 and other tachykinin receptors is mediated through a Gq-dependent activation of phospholipase C (PLC) (Khawaja and Rogers, 1996; Maggi, 1995; Nakanishi *et al*, 1993; Shigemoto *et al*, 1990) that causes phosphoinositol 4, 5 biphosphate (PIP_2) breakdown into 1,4,5 inositol triphopshate (IP_3) and diacylglycerol (DAG). This leads to Ca^{2+} mobilization and induction of Ca^{2+} dependent downstream signaling pathways, mediating physiological processes such as smooth muscle contraction and neuronal depolarization (Maggi, 1995). Activation of adenylyl cyclase and formation of cAMP can also occur through NK-3 receptor activation, although this pathway is much less efficient than PLC activation (Nakanishi *et al*, 1993).

In summary, the NK-3 receptor is a G-protein coupled receptor and has unique features that determine its interaction with endogenous and synthetic ligands. Upon NK-3 receptor activation, there is a Gq-dependent stimulation of phospholipase C which causes generation of 1,4,5 inositol triphopshate (IP_3) and diacylglycerol (DAG) leading to Ca^{2+} mobilization and induction of Ca^{2+} dependent downstream signaling pathways. The NK-3 receptor and the other mammalian tachykinin receptors, NK-1, NK-2, share moderate similarities in amino acid sequences in their transmembrane domains, however they diverge in the sequence of both their amino and carboxyl terminus, which may have functional significance in ligand recognition and signal transduction, respectively.

Endogenous ligands for the NK-3 receptor

The endogenous mammalian tachykinins include substance P, neurokinin-A (NK-A), neurokinin-B (NK-B), neuropeptide-K (NPK), and neuropeptide-γ (NP-γ), forms of

NK-A in which the amino terminus peptide sequence is extended (Krause *et al*, 1990). Evidence from ligand binding studies show that NK-B has greater affinity for the NK-3 receptor. However, NK-A and substance P can also bind to and activate the NK-3 receptor under physiological conditions (Krause *et al*, 1990). In the following paragraphs, the regulation of the production of the mammalian endogenous ligands substance P, NK-A and NK-B will be considered but with emphasis on NK-B as the endogenous ligand for NK-3 receptors.

There are three precursor mRNAs for substance P, α, β and γ-preprotachykinin A, and two precursor mRNAs for NK-A, β and γ-preprotachykinin A (Figure 1.2). The three mRNAs are all transcribed from a single gene, preprotachykinin A gene (Kotani *et al*, 1986; Krause *et al*, 1990). The unprocessed precursor mRNA is composed of seven exons and interrupted by six introns (Kotani *et al*, 1986). Substance P is encoded in part of exon three, whereas NK-A is encoded by exon six (Kotani *et al*, 1986; Krause *et al*, 1990). Production of the precursor mRNAs is regulated in a tissue-specific manner by alternative RNA splicing through either the inclusion or exclusion of the sixth and fourth exon (Kotani *et al*, 1986; Krause *et al*, 1990).

In contrast to substance P and NK-A, there is only one precursor mRNA for NK-B that is encoded by a single preprotachykinin B gene (Kotani *et al*, 1986). The preprotachykinin B mRNA is encoded by seven exons, with exons two to six encoding the precursor peptide, protachykinin B. Exon two encodes the sequence corresponding to a signal peptide common to secretory proteins, and exon five encodes NK-B (Kotani *et al*, 1986). Within protachykinin B, the unprocessed NK-B is flanked on both sides by a

pair of basic amino acids, lysine and arginine, recognition sites for subsequent proteolytic cleavage (Kotani *et al*, 1986).

Neurokinin-B and NK-3 receptor expression in the basal ganglia and associated regions in the brain

Anatomical studies provide evidence in support of NK-B and NK-3 receptor expression in brain regions pertinent to behavioral activity and neuronal plasticity induced by drugs of abuse. These regions include the frontal and anterior cingulate cortices, caudate putamen, nucleus accumbens, globus pallidum, ventral pallidum, substantia nigra and ventral tegmental area. Expression of NK-B and NK-3 receptor can be further localized to either nerve terminals or perikarya in these brain regions.

Both NK-3 receptor and NK-B are co-expressed in perikarya and nerve fibers in layers IV and V of the cortex, nucleus accumbens, caudate putamen, substantia nigra, ventral tegmental area, and the globus pallidum to a lesser extent (Ding *et al*, 1996; Langlois *et al*, 2001; Marksteiner *et al*, 1992a; Marksteiner *et al*, 1992c; Mileusnic *et al*, 1999; Saffroy *et al*, 1988; Shughrue *et al*, 1996; Warden and Young, 1988), which suggest that perikarya in those regions produce NK-B, and the neurons present may also be modulated by NK-3 receptor activity. It also implies that the neurons contained in the areas to which NK-B containing neurons project may express NK-3 receptors and may be responsive to NK-B.

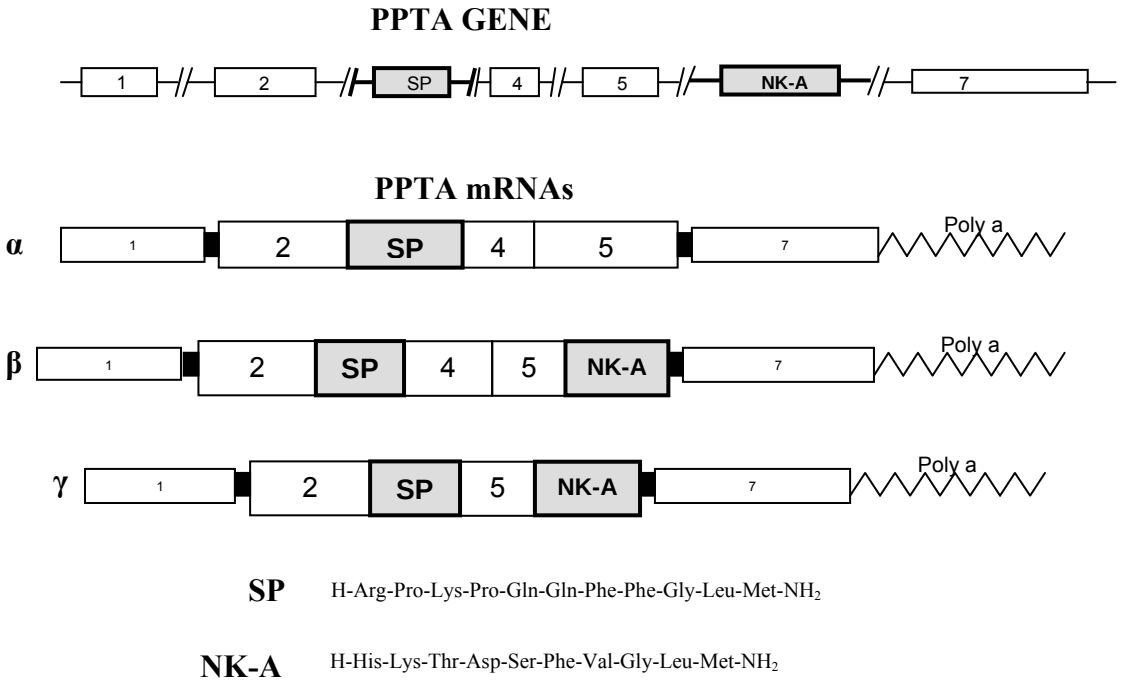

Figure 1.2: Structures of the three preprotachykinin A precursor mRNAs, α, β and γ and the preprotachykinin A (PPTA) gene. The SP and NK-A coding exons are indicated within the gene and mRNA structures, and their peptide sequences are also shown. (Adapted from Kotani, 1986).

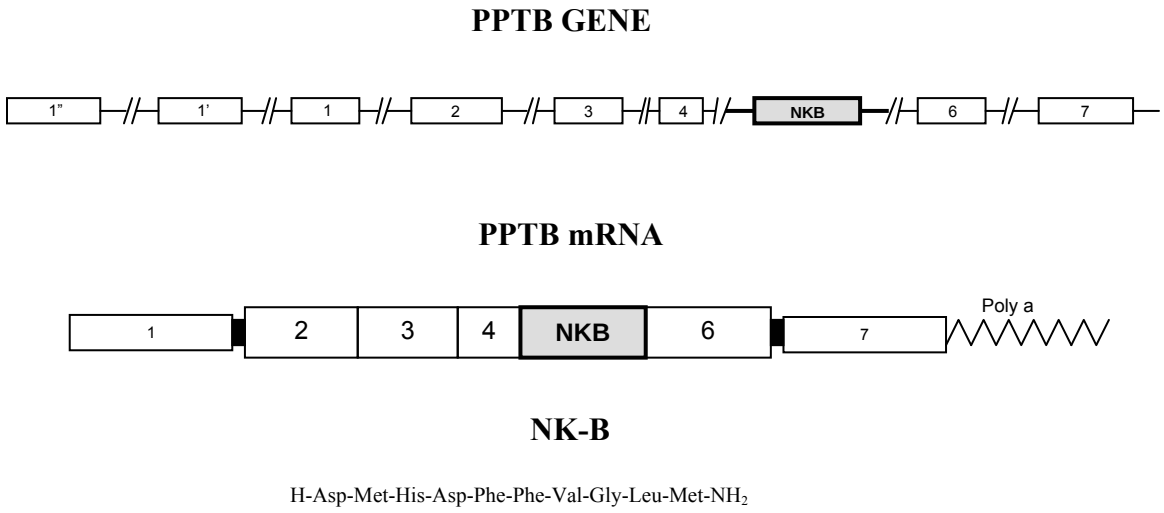

Figure 1.3: Structures of the mRNA preprotachykinin B (PPTB) and gene, precursors to neurokinin-B (NK-B). Within the mRNA structures, the protein-coding and the untranslated regions are indicated by large and small boxes, respectively. The numbered boxes and those indicated as NK-B stand for corresponding numbered exons and for exons encoding NK-B in exon five. The peptide sequence of NK-B is also shown (Adapted from Kotani, 1986).

In comparison, little or no NK-3 receptor immunoreactivity is detected in the ventral pallidum, subthalamic nucleus and thalamus (Ding *et al*, 1996; Shughrue *et al*, 1996), but there are extensive NK-B immunoreactive fibers present (Marksteiner *et al*, 1992a; Marksteiner *et al*, 1992c; Shughrue *et al*, 1996). This pattern of immunoreactivity may indicate that these areas may have other tachykinin receptors and may be responsive to NK-B. In addition, perikarya of the ventral pallidum are also immunoreactive for NK-B suggesting that these neurons produce NK-B. Therefore, there is differential localization of NK-3 receptor and the endogenous peptide, NK-B, in the brain. The presence or absence of NK-B and the NK-3 receptor in perikarya and/or fibers in different regions of the brain may have functional implications about possible sites of NK-3 receptor activation and NK-B production.

A number of studies have used combined retrograde labeling and fluorescence to further characterize neurons in the striatum that produce NK-B and their possible projections to other brain regions. In the lateral stripe of the dorsal striatum, neurons immunoreactive for NK-B and its precursor peptide, preprotachykinin B (PPTB), constitute only 5% of striatal neurons, and are not immunoreactive for either choline acetyltransferase, somatostatin, calretinin or parvalbumin (Furuta *et al*, 2000). Twenty-five percent of NK-B producing neurons in the caudate putamen also express the peptides prodynorphin, 65% express protachykinin A, and 30% express proenkephalin. As compared to the caudate putamen, in the ventral striatum, which contains the nucleus accumbens and olfactory tubercle, NK-B-containing neurons comprise a higher population of neurons, 11%. Similar to the caudate putamen, no NK-B neurons in the nucleus accumbens showed immunoreactivity for interneuron markers. However, there is

a higher percentage of co-expression of prodynorphin and protachykinin A with NK-B in the nucleus accumbens core and shell than in the caudate putamen (Furuta *et al*, 2000; Furuta *et al*, 2002; Marksteiner *et al*, 1992c). For instance, in the nucleus accumbens core, 80% of PPTB neurons also have prodynorphin, and 83% have protachykinin A. In the accumbens shell, PPTB-immunoreactive neurons were less frequently immunoreactive for protachykinin A (47-50%) and prodynorphin (61-62%) (Furuta *et al*, 2002). There is less co-expression of proenkephalin with NK-B in the nucleus accumbens (5%) than in the caudate putamen (30%) (Furuta *et al*, 2000; Furuta *et al*, 2002). Injection of fluoro-gold into NK-B immunoreactive regions in the substantia innominata and its sublenticular part retrogradely labeled connecting clusters of neurons in the nucleus accumbens and neurons in the lateral stripe of the striatum and caudate putamen (Furuta *et al*, 2000; Zhou *et al*, 2004). These studies demonstrate that NK-B containing neurons in the striatum project mainly to basal forebrain structures such as the substantia innominata.

Neurons in the substantia innominata have been shown to be modulated by NK-3 receptor activity (Furuta and Kaneko, 2006). NK-3 receptor immunoreactivity is detected in GABAergic neurons in the substantia innominata, ventral pallidum and external segment of globus pallidum, and also in cholinergic neurons in the horizontal limb of the diagonal band of Broca and medial septum (Furuta *et al*, 2006; Furuta *et al*, 2004). The GABAergic neurons of substantia innominata that express NK-3 receptors project to the cortex. Injection of wheat germ agglutinin into the motor cortex retrogradely labels neurons in the substantia innominata, ventral pallidum and external segment of globus pallidum of which 27% are NK-3 receptor immunoreactive. The retrogradely labeled

neurons of the basal forebrain regions also slowly depolarize and have increased action potentials in response to the NK-3 receptor agonist senktide (Furuta *et al*, 2006; Furuta *et al*, 2004), suggesting that GABAergic neurons in the basal forebrain are modulated by NK-B release from the striatum. The activated neurons in the basal forebrain that have been shown to innervate inhibitory cortical neurons, disinhibit them, thereby facilitating cortical neurotransmission (Furuta *et al*, 2006).

In the dorsal striatum, a majority of the NK-B neurons (86%) express dopamine D1 receptor mRNA, and 85 and 89% of NK-B neurons have dopamine D1 receptor mRNA in the nucleus accumbens and in the lateral stripe of the striatum respectively (Sonomura *et al*, 2007). Downstream signaling molecules for dopamine D1 receptors such as DARPP-32 and the protein kinase A substrate striatal-enriched tyrosine phosphatase (STEP) are also co-expressed with NK-B in the striatum. NK-B containing neurons in the caudate putamen are not found to express DARPP-32, but DARPP-32 immunoreactivity is present in 47% of NK-B neurons in the nucleus accumbens core and in 22% of PPTB immunoreactive neurons in the shell (Furuta *et al*, 2000). The NK-B neurons in the caudate putamen are immunoreactive for STEP (Sonomura *et al*, 2007). Therefore, these findings suggest that dopaminergic neurotransmission in the striatum may modulate function of NK-B producing neurons (Sonomura *et al*, 2007).

There is evidence that chronic depression of dopaminergic transmission alters expression of NK-B. 6-OHDA injections into the substantia nigra and ventral tegmental area leads to a loss of tyrosine hydroxylase mRNA, and a compensatory increase in both the number of neurons containing NK-B mRNA and NK-B transcript levels ipsilateral to the lesion. On the contralateral side, the number of neurons with NK-B mRNA and

transcript levels are decreased (Burgunder and Young, 1989). In addition, chronic treatment with the antipsychotic agent haloperidol for 10 days also increases NK-B mRNA transcript levels and number of neurons containing NK-B in the dorsal and ventral striatum (Marksteiner *et al*, 1992b). These findings suggest that dopaminergic input from the substantia nigra and the VTA may have an inhibitory influence on expression of NK-B in the striatum.

In summary, various anatomical studies provide evidence in support of NK-B and NK-3 receptor expression in brain regions pertinent to locomotive behavior and neuronal plasticity. In the striatum, a subpopulation of GABAergic medium sized neurons express both NK-B and dopamine D1 receptors and are thought to project mainly to the substantia innominata (Sonomura *et al*, 2007). Functionally, activation of NK-3 receptors expressed on neurons in the substantia innominata disinhibits cortical interneurons, and thereby facilitates cortical neurotransmission. Dopaminergic transmission to the striatum has been shown to modulate function of striatal NK-B producing neurons and their projections to the basal forebrain, however the mechanism by which it occurs is currently unknown.

Regulation of dopamine-mediated synaptic transmission and behaviors by NK-3 receptors

Studies demonstrate the localization of NK-3 receptors on dopaminergic neurons in the substantia nigra and ventral tegmental area (Bannon *et al*, 1995; Chen *et al*, 1998; Lessard *et al*, 2007), although NK-3 receptors are also present on non-dopaminergic neurons in these regions (Lessard *et al*, 2007). Electron microscopy shows NK-3

receptors contained in somata and large dendritic areas in dopaminergic neurons in the substantia nigra and ventral tegmental area, often within areas involved in intracellular trafficking such as Golgi lamellae (Lessard *et al*, 2007). In addition, there is evidence of novel nuclear targeting of NK-3 receptors in neurons in the ventral tegmental area (Lessard *et al*, 2009). Overall, these studies demonstrate that in the substantia nigra and ventral tegmental area, NK-3 receptors are present in both dopaminergic as well as non-dopaminergic neurons.

Functional studies demonstrate that NK-3 receptor activity alters dopaminergic neuronal activity, neurochemistry, and behaviors. Studies have shown that activation of NK-3 receptors in the ventral tegmental area and substantia nigra by the NK-3 receptor agonist senktide stimulates dopaminergic neuronal activity *in vivo* and *in vitro*, including increases in action potentials and firing rates (Keegan *et al*, 1992; Overton *et al*, 1992). Activation of NK-3 receptors also increases dopamine release and tissue content of dopamine metabolites in the dorsal striatum, nucleus accumbens and prefrontal cortex (Bannon *et al*, 1995; Humpel *et al*, 1991; Marco *et al*, 1998). Dopamine release measured by voltammetry in the dorsal striatum, nucleus accumbens, or prefrontal cortex was increased after local administration of senktide into the substantia nigra par compacta or ventral tegmental area. This release is blocked by pretreatment with the NK-3 receptor antagonist SR 142801 (Marco *et al*, 1998). Nigral infusions of senktide increase DOPAC tissue concentration in the dorsal striatum in rats (Bannon *et al*, 1995; Humpel *et al*, 1991). NK-3 receptor activation in the substantia nigra and ventral tegmental area elicits dopamine-mediated behaviors (Deschamps and Couture, 2005; Elliott *et al*, 1991; Jocham *et al*, 2007; Placenza *et al*, 2004; Stoessl *et al*, 1991). For example,

microinjections of senktide into the ventral tegmental area or substantia nigra elicit behaviors such as locomotion (Elliott *et al*, 1991; Placenza *et al*, 2004; Stoessl *et al*, 1991), rearing, sniffing, digging, face washing, head scratching and wet dog shakes (Deschamps *et al*, 2005; Stoessl *et al*, 1991). In addition to being blocked by the NK-3 receptor antagonist SB 235378, senktide-induced locomotion, wet dog shakes, rearing, and sniffing are attenuated by administration of the D1 receptor antagonist SCH 23390 (Deschamps *et al*, 2005; Placenza *et al*, 2004). Conversely, activation or blockade of NK-3 receptors modulates dopamine receptor agonist-induced behaviors (Bishop *et al*, 2004; Jocham *et al*, 2007; Jocham *et al*, 2006; Placenza *et al*, 2004). Locomotion and stereotypy induced by dopamine D1 agonist SKF 82958 or by cocaine is attenuated by infusion of the NK-3 receptor antagonist SR 142801 into the substantia nigra or systemic administration (Bishop *et al*, 2004; De Souza Silva *et al*, 2006b; Jocham *et al*, 2006). In addition, cocaine-induced hyperactivity is potentiated by NK-3 receptor agonist administration (Jocham *et al*, 2007). Cocaine-seeking behaviors can be reinstated after infusion of the substance P analogue DiMe-C7 acting on NK-3 receptors in the ventral tegmental area (Placenza *et al*, 2004). In conclusion, NK-3 receptors are implicated in modulating dopaminergic neuronal function and, in particular, cocaine-induced hyperactivity and cocaine-seeking behaviors.

NK-3 receptors as a therapeutic target for CNS disorders

A very small number of epidemiological studies have examined genetic associations of CNS disorders with the NK-3 receptor gene (TACR3). In a 2008 study by Foroud and colleagues a significant association of several SNPs in the TACR3 gene with

phenotypes of cocaine and alcohol co-abuse was reported. In another study, an association with schizophrenia was not found in a Japanese population (Saito *et al*, 2008).

The NK-3 receptor is currently studied as a potential therapeutic target for treatment of various neuropsychiatric illnesses such as schizophrenia, mood disorders and Parkinsonism (Panocka *et al*, 2001; Ribeiro and De Lima, 1998; Spooren *et al*, 2005). Currently, the therapeutic potential of NK-3 receptor antagonists is being scrutinized in clinical trials. In a small randomized double blinded placebo-controlled clinical trial, it is reported that treatment with the NK-3 receptor antagonist SR 142801 did not improve levadopa-induced dyskinesias after administration of levadopa in Parkinson's disease patients (Mesnage *et al*, 2004). In another clinical trial, SR 142801 was tested as a novel anti-psychotic agent in the treatment of schizophrenia and schizoaffective disorder. Patients received once daily treatment of either haloperidol or SR 142801 for 6 weeks. Patients receiving SR 142801 showed modest improvement in severity of illness, psychosis, and in positive and negative symptoms, however in comparison to haloperidol SR 142801 was less efficacious (Meltzer *et al*, 2004). The effects of SR 142801 for the treatment of panic disorder were assessed in a small pilot study. In response to panic provoked by cholecystokinin tetrapeptide, patients received either placebo or SR 142801. SR 142801 had no effect on panic symptoms as compared to placebo (Kronenberg *et al*, 2005). Although a small number of clinical trials have been conducted so far, NK-3 receptors continue to be studied in their potential role in CNS disorders, and compounds that antagonize NK-3 receptors are currently being characterized in preclinical and clinical studies.

Pharmacological properties of cocaine

Cocaine inhibits the re-uptake of the monoamines dopamine, serotonin and norepinephrine (Li *et al*, 1996; Reith *et al*, 1997; Ritz *et al*, 1988). The inhibition of monoamine re-uptake by cocaine is a direct result of its binding to the dopamine (DAT), norepinephrine (NET) and serotonin (SERT) transporters (Li *et al*, 1996; Reith *et al*, 1997). In addition to inhibiting reuptake, cocaine also has local anesthetic properties that are due to the inhibition of Na^+ ion channels (Reith *et al*, 1985).

The primary psychoactive effects of cocaine are a result of its actions on the dopamine transporter (Ritz *et al*, 1987). The dopamine transporter protein is a member of the large family of Na^+/ Cl^- dependent transporters that also include the norepinephrine and serotonin transporters. It is expressed in presynaptic nerve terminals of dopaminergic neurons in the nucleus accumbens and caudate putamen (Giros *et al*, 1996), and plays a crucial role in regulating dopaminergic neurotransmission in the striatum through regulating dopamine clearance from the synapse (Giros *et al*, 1996). Administration of cocaine directly into the nucleus accumbens and caudate putamen increases extracellular dopamine (Bradberry and Roth, 1989; Carboni *et al*, 1989; Li *et al*, 1996; Nicolaysen and Justice, 1988; Reith *et al*, 1997), and to a lesser extent serotonin and norepinephrine (Li *et al*, 1996; Reith *et al*, 1997). Consistent with its mechanism in blocking dopamine reuptake, cocaine's effect is both dependent on neuronal firing and extracellular Ca^{2+}, as shown by abolishment of cocaine-induced increase in dopamine levels after inhibition of neuron activity with gamma-butyrolactone, or after Ca^{2+} depletion (Carboni *et al*, 1989).

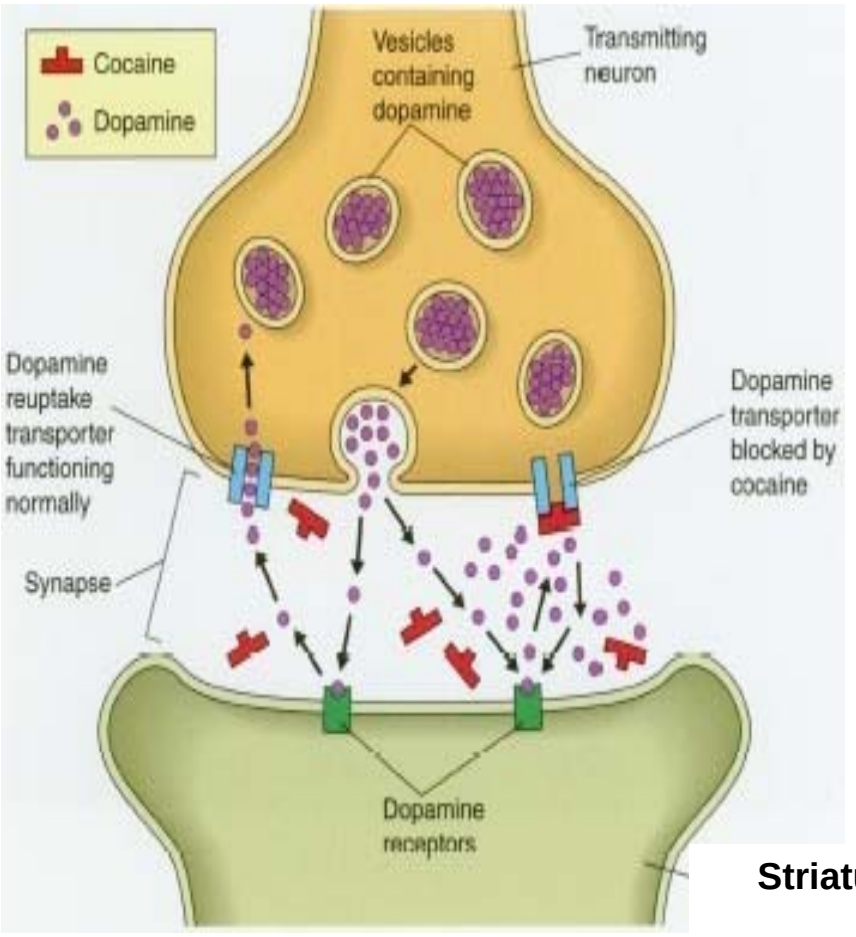

Figure 1.4: Mechanism of action of cocaine. Binding of cocaine to neurotransmitter transporter proteins inhibits reuptake of neurotransmitters (dopamine, serotonin and norepinephrine) into presynaptic terminals. Adapted from www.humanillness.com.

Mechanism of action of cocaine as related to its behavioral effects

Cocaine inhibition of dopamine re-uptake through the dopamine transporter is functionally coupled to its behavioral effects which include locomotor hyperactivity, stereotypy, and drug reinforcement. There is a strong correlation between cocaine binding and induction of stereotypic behaviors (Reith, 1986). Deletion of the dopamine transporter gene in a knockout mouse model also shows that locomotion and stereotypy induced by cocaine are primarily due to its blockade of the dopamine transporter. High doses of cocaine that would cause significant locomotor hyperactivity and stereotypies in a wild-type mouse produce no locomotor or stereotypic effects in the dopamine transporter knockout mouse (Giros *et al*, 1996). Additionally, in drug self-administration, a behavioral model that is used to study compulsive drug taking, cocaine analogs were studied to determine if they would be substituted for cocaine. Analogs that potently inhibit [^{3}H] mazindol binding to the dopamine transporter are potent substitutes for cocaine (Ritz *et al*, 1988). These findings demonstrate that cocaine binding to the dopamine transporter is directly correlated with its acute psychomotor and reinforcing effects.

Other behavioral effects of cocaine may be mediated by other signaling systems. For instance, the rewarding effects of cocaine may involve the dopamine transporter and also the serotonin transporter (Sora *et al*, 2001). In studies of cocaine reward as assessed by the conditioned place preference procedure, both DAT and SERT double knockout ($DAT^{-/-}$ and $SERT^{-/-}$) mice and DAT knockout-SERT heterozygous ($DAT^{-/-}$ and $SERT^{+/-}$) mice do not show a place preference for cocaine. Interestingly, DAT knockout ($DAT^{-/-}$) mice still show a place preference, which suggests that the absence of the dopamine

transporter is not sufficient to prevent cocaine reward, and may require partial deficiency of the serotonin transporter (Sora *et al*, 2001).

In addition to its acute psychomotor effects, upon repeated intermittent exposure, cocaine induces behavioral sensitization that is characterized as a progressively heightened behavioral activity after re-exposure (Post and Rose, 1976). This sensitization appears to persist after long periods of drug abstinence (Heidbreder *et al*, 1996). Behavioral sensitization is thought to result as a consequence of neuroplastic changes induced by cocaine (Robinson and Berridge, 2001) that may involve other receptor signaling systems in addition to dopamine. Studies have shown that glutamatergic signaling through NMDA receptors, mu and kappa opioid receptor systems in addition to dopamine receptors also play a role in the induction of behavioral sensitization to cocaine (Hummel *et al*, 2006; Schroeder *et al*, 2007; Shippenberg and Rea, 1997; Vanderschuren and Kalivas, 2000).

In conclusion, cocaine's behavioral effects such as locomotor hyperactivity, stereotypy, reinforcement, drug reward and behavioral sensitization are primarily mediated by inhibition of dopamine re-uptake in presynaptic terminals, but may involve more complex interactions between dopaminergic and other receptor systems in the brain. Drug-induced behavioral sensitization results from alterations in the mesocorticolimbic pathway, a pathway also involved in drug-reward, and these drug-induced neuroadaptations may contribute to relapse to cocaine seeking behavior (Robinson *et al*, 2001).

Cellular localization of dopamine receptors in the striatum

Dopamine released from nerve terminals in the striatum activates two types of dopamine receptors, dopamine D1-like and D2-like receptors, which have distinct signal transduction mechanisms and anatomical localizations. Activation of dopamine D1-like receptor (D1 and D5) in the striatum stimulates adenylyl cyclase activity via stimulatory G-proteins (Gs/Golf) causing increased cAMP formation, whereas dopamine D2-like receptor (D2, D3 and D4) activation inhibits adenylyl cyclase activity via Gi/Go proteins to decrease cAMP formation (O'Boyle *et al*, 1989; Stoof and Kebabian, 1981).

Dopamine D1 and D2 receptors have distinct expression in the striatum. In situ hybridization studies show that 48% of neurons in the striatum contain dopamine D1 receptor mRNA, and 43% have dopamine D2 receptor mRNA (Le Moine *et al*, 1991). The dopamine D1 receptor is expressed predominantly by GABAergic medium sized neurons which also express substance P and dynorphin (Gerfen *et al*, 1990; Le Moine *et al*, 1991). The dopamine D2 receptor is expressed within the striatum by medium sized neurons that produce enkephalins, and also by cholinergic interneurons (Le Moine *et al*, 1991). The two major efferent pathways from the striatum, one to the substantia nigra and entopeduncular nucleus, and the other to the globus pallidus in rodents are mostly comprised of neurons expressing the dopamine D1 receptor and dopamine D2 receptor, respectively (Albin *et al*, 1989). Studies using fluorescent retrograde tracing combined with in situ hybridization identify dopamine D1 receptor containing striatal neurons as neurons that project to the substantia nigra, and dopamine D2 receptor containing neurons projecting to the globus pallidum (Gerfen *et al*, 1990; Gerfen and Young, 1988).

The role of dopamine receptors in behavioral effects of cocaine

Both dopamine D1 and D2 receptors are involved in the acute effects of cocaine that include hyperlocomotion and stereotypy. Pretreatment with either the dopamine D1 receptor antagonist SCH 23390 or the dopamine D2 receptor antagonist haloperidol attenuates cocaine-induced locomotion and stereotypy (Cabib *et al*, 1991; Mattingly *et al*, 1996). Other behavioral effects of cocaine appear to be differentially mediated by dopamine D1 and D2 receptors. For instance, although both receptors appear to be involved in behavioral sensitization to cocaine, dopamine D2 receptor activity may be necessary in the development (Mattingly *et al*, 1996; Tella, 1994), and dopamine D1 receptors in the expression of behavioral sensitization to cocaine (Pierce and Kalivas, 1997). Dopamine D1 receptors play a role in the acquisition of cocaine reward as assessed by the conditioned place preference procedure. Administration of the dopamine D1 receptor antagonist SCH 23390 prior to repeated cocaine blocks the development of a place preference (Cervo and Samanin, 1995). Cocaine-seeking behavior appears to be mediated by dopamine D2 receptors, and not dopamine D1 receptors. In a self-administration and reinstatement model, administration of dopamine D2-like receptor agonists reinstated cocaine-seeking behavior (Dias *et al*, 2004). Overall, these studies suggest that both dopamine D1 and D2 receptors mediate acute locomotion and stereotypy induced by cocaine. Other behaviors appear to be more complex in nature involving either dopamine D1 or D2 receptors as in cocaine-seeking behavior or involve both in temporally distinct processes as is the case of behavioral sensitization.

Down stream effector of dopamine receptors associated with cocaine: Glycogen synthase kinase 3

Activation of dopaminergic neurotransmission in the striatum can lead to the induction of cAMP-dependent and cAMP-independent signaling cascades involving a host of protein and effector molecules. Glycogen synthase kinase 3 (GSK3) is an effector molecule in dopaminergic signaling that also has a role in mediating behavioral effects of cocaine. GSK3 has two isoforms in the brain, GSK3α and GSK3β (Woodgett, 1990), both of which are primarily regulated by phosphorylation at serine 21 and serine 9 residues, respectively, leading to inhibition of activity (Grimes and Jope, 2001b). The α isoform of GSK3 is distinguished from the β isoform by a glycine-rich extension at the amino terminus (Lee *et al*, 2007). Activity of GSK3 α and β is also regulated by tyrosine phosphorylation at tyrosine 279 and tyrosine 216 residues respectively leading to activation, and by protein complex formation and intracellular localization (Grimes *et al*, 2001b). Phosphorylation of GSK3 at its serine residues has been shown to be regulated by protein kinase B/Akt, and also by DARPP-32, P70 S6 kinase, MAP kinase-activated protein kinase-1 (MAPKAP-1), protein kinase A, and protein kinase C (Alessi *et al*, 1996; Grimes *et al*, 2001b; Li *et al*, 2000; Svenningsson *et al*, 2003). Most substrates that GSK3 phosphorylates are primed by prior phosphorylation by another kinase, with the exception of the transcription factor myc (Grimes *et al*, 2001b). Downstream substrates of GSK3 include structural proteins, signaling and metabolic proteins, and most notably transcription factors (Grimes *et al*, 2001b). These include cAMP-dependent responsive element binding protein (CREB) (Fiol *et al*, 1994; Grimes and Jope, 2001a), cmyc (Plyte

et al, 1992), AP-1 and cjun (Boyle *et al*, 1991), and beta catenin (Seeling *et al*, 1999). In most cases, GSK3 activity is inhibitory towards transcription factor activation, and many of the transcription factor substrates are involved in neuronal survival, apoptosis and neuronal plasticity (Grimes *et al*, 2001b).

GSK3 has a role in mediating dopamine and psychostimulant-induced behaviors. There is evidence that inhibition of GSK3 activity by structurally unrelated pharmacological inhibitors lithium, alsterpaullone, TDZD, indirubin-3-monoxime, sodium valproate and SB 216763 attenuate dopamine-mediated behaviors (Beaulieu *et al*, 2004; Miller *et al*, 2009). Administration of GSK3 inhibitors significantly attenuate horizontal activity, vertical activity and stereotypy in dopamine transporter knockout mice (Beaulieu *et al*, 2004), and also attenuate dopamine D1 receptor stimulated hyperactivity(Miller *et al*, 2009) Inhibition of GSK3 by the selective inhibitor SB 216763 attenuates cocaine-induced hyperactivity, development of behavioral sensitization and cocaine reward (Miller *et al*, 2009). While homozygous knockout of the GSK3β gene results in embryonic lethality, partial genetic deletion of GSK3β in heterozygous mice results in reduced behavioral responses to acute amphetamine administration as compared to wild-type mice (Beaulieu *et al*, 2004). Conversely, overexpression and activation of GSK3β in a transgenic mouse model results in overall heightened locomotor activity and lack of habituation to a novel environment (Prickaerts *et al*, 2006).

The regulation of GSK3 phosphorylation following acute administration of psychostimulants is currently studied. Increases in phosphorylation of GSK3β in the striatum 15 mins after acute amphetamine and cocaine have been reported (Svenningsson *et al*, 2003). A decrease in GSK3β phosphorylation has been detected 30 to 90 mins after

administration of amphetamine in the striatum (Beaulieu *et al*, 2004), and 30 mins after a cocaine injection in the caudate putamen (Miller *et al*, 2009). The increase in GSK3β phosphorylation in the striatum 15 mins after amphetamine or cocaine is accompanied by an increase in DARPP-32 phosphorylation at its threonine 34 residue, and also increased Akt phosphorylation at the threonine 308 residue (Svenningsson *et al*, 2003). A reduction of Akt and GSK3α and GSK3β phosphorylation in the striatum 30 mins to 90 mins after amphetamine administration (Beaulieu *et al*, 2004) and 30 mins after a cocaine injection in the caudate putamen (Miller *et al*, 2009) have also been reported. In addition, studies in dopamine transporter knockout mice show decreased Akt phosphorylation at threonine-308 and a decrease in phosphorylation of GSK3α and β in the striatum, which may reflect a compensatory state in the DAT knockout mouse (Beaulieu *et al*, 2004).

In summary, GSK3 has a crucial role in the regulation of gene expression, neural plasticity, cell survival and apoptosis. In addition, GSK3 activity has been shown to modulate dopamine- and psychostimulant-induced behaviors. A growing number of studies demonstrate changes in GSK3 phosphorylation after acute administration of psychostimulants. These reports share the same overall hypothesis that transient changes in GSK3 phosphorylation in the striatum play a role in psychostimulant-induced behaviors.

General summary of objectives

The lack of effective FDA-approved pharmacotherapies for cocaine addiction has driven investigators to search for novel therapeutic targets. Recently, the NK-3 receptor has been proposed as such a target based on preclinical studies demonstrating that

neurokinin-3 receptor activation or blockade alters dopaminergic function and behavior, and in particular modulates cocaine-induced behaviors. With the primary intent of characterizing a novel therapeutic target, studies presented in this dissertation examine acute and long-term effects of NK-3 receptor blockade on cocaine-induced hyperactivity and behavioral sensitization to cocaine. Possible mechanisms of NK-3 receptor antagonism were investigated including changes in dopamine D1 receptor expression and GSK3 phosphorylation.

CHAPTER 2

BLOCKADE OF TACHYKININ-3 RECEPTORS MODULATES DOPAMINE-MEDIATED BEHAVIORAL HYPERACTIVITY

Introduction

The mammalian tachykinin family of neuropeptides that includes substance P, neurokinin A, neurokinin B, neuropeptide K and neuropeptide gamma exert their biological actions through G-protein coupled receptors termed NK-1, NK-2, and NK-3 (Maggi, 1995; Massi *et al*, 2000). Activation of NK-3 receptors primarily causes phosphoinositol 4, 5 biphosphate (PIP_2) breakdown into 1,4,5 inositol triphosphate (IP_3) and diacylglycerol through phospholipase C activation (Khawaja *et al*, 1996; Maggi, 1995), eventually leading to Ca^{2+} mobilization and induction of Ca^{2+}dependent downstream signaling pathways.

NK-3 receptors are found on peripheral nerve endings of primary afferent neurons innervating the respiratory, gastrointestinal and urinary tracts (Patacchini and Maggi, 2001), however they are also differentially expressed in the central nervous system. NK-3 receptors localized in the substantia nigra, ventral tegmental area (VTA) and prefrontal cortex (Dam *et al*, 1990) are found to regulate dopaminergic neurotransmission and locomotive behaviors (Elliott *et al*, 1991; Overton *et al*, 1992). Tyrosine hydroxylase-containing neurons in the substantia nigra and VTA have been shown to express NK-3 receptors (Chen *et al*, 1998). Activation of NK-3 receptors in the substantia nigra and VTA stimulates dopaminergic neuronal activity (Keegan *et al*, 1992; Overton *et al*, 1992), and increases dopamine release and metabolism in the nucleus accumbens,

striatum, and prefrontal cortex (Bannon *et al*, 1995; Humpel *et al*, 1991; Marco *et al*, 1998). In addition, NK-3 receptor activation elicits behaviors such as locomotion, rearing, sniffing and wet dog shakes in rats (Deschamps *et al*, 2005; Elliott *et al*, 1991; Jocham *et al*, 2007; Stoessl *et al*, 1991), which are diminished by administration of the dopamine D1 receptor antagonist SCH 23390 (Deschamps *et al*, 2005). In addition, NK-3 receptor activation potentiates locomotion induced by cocaine in rodents (Jocham *et al*, 2007) and also decreases exploratory activity, aerial scanning, and terrestrial glancing by cocaine in responsive non-human primates (de Souza Silva *et al*, 2006a). Conversely, dopamine D1 receptor-mediated and cocaine-induced behaviors are attenuated after administration of NK-3 receptor antagonists in rats (Bishop *et al*, 2004; Jocham *et al*, 2006) and in cocaine responsive non-human primates (De Souza Silva *et al*, 2006b). These findings demonstrate that NK-3 receptors modulate dopaminergic function and behavior.

To date, the majority of studies on NK-3 receptors and dopamine have examined modulation of dopaminergic function after acute administration of NK-3 receptor agonists and antagonists. However, effects of prior repeated blockade of NK-3 receptors on dopaminergic function have not been investigated. Since acute blockade of NK-3 receptors diminishes dopamine-mediated function and behavior, we hypothesized that repeated NK-3 receptor blockade may result in dopamine receptor super-sensitivity and enhancement of behaviors. Supporting evidence shows that dopaminergic behaviors are enhanced after previous chronic administration of dopamine receptor antagonists, and the change in behaviors results from up-regulation of dopamine receptors in the striatum (Hess *et al*, 1986; Hess *et al*, 1988). In particular, we propose that dopaminergic

behaviors and striatal dopamine D1 receptors may be altered after repeated NK-3 receptor blockade because it has been previously shown that NK-3 receptor antagonists attenuate dopamine D1 receptor-mediated behavior (Bishop *et al*, 2004), and conversely administration of dopamine D1 receptor antagonists attenuate behaviors induced by NK-3 receptor agonists (Deschamps *et al*, 2005). Therefore, the objectives of the present study were to investigate the effects of acute and repeated NK-3 receptor blockade on subsequent behavioral responses to cocaine and to the selective dopamine D1 receptor agonist SKF 82958, and to examine changes in striatal dopamine D1 receptors that possibly mediate the change in behavioral response.

Materials and methods

Animals

Adult male CD-1 mice (Charles River Laboratories, Raleigh, NC, USA) were group-housed (4-6 per cage) in a temperature and humidity controlled environment on a 12-h light–dark cycle (lights on at 7AM) with *ad libitum* access to food and water. Animals were handled daily prior to the beginning of experiments. All experiments were conducted in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory animals and with approval from Temple University School of Medicine Institutional Animal Care and Use Committee.

Drugs and chemicals

Cocaine hydrochloride was generously provided by the National Institute on Drug Abuse and dissolved in a sterile 0.9% saline solution. The dopamine D1 receptor agonist 6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (SKF

82958) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in sterile saline. The NK-3 receptor antagonist (*S*)-3-methyl-2-phenyl-N-(1-phenylpropyl)-4-quinolinecarboxamide (SB 222200) was also obtained from Sigma-Aldrich and dissolved in a vehicle composed of 60% polyethylene glycol (PEG-200) and 40% distilled water. [^{3}H] SCH 23390 (84 Ci/mmol) was obtained from Perkin Elmer (Waltham, MA, USA). Fluphenazine and mianserin were both obtained from Sigma-Aldrich. Cocaine and SKF 82958 were injected intraperitoneally at volumes of 3 ml/kg, and SB 222200 was injected subcutaneously at a volume of 2 ml/kg.

Drug administration and behavioral assessment

The 4-quinolinecarboxamide based compound SB 222200 was used in our study to antagonize NK-3 receptors. It has been shown to be a centrally active compound with 57-fold selectivity for NK-3 versus NK-2 receptors and 100,000-fold selectivity for NK-3 versus NK-1 receptors (Sarau *et al*, 2000). The doses of SB 222200 and pretreatment time used were chosen based on the *in vivo* pharmacological properties of SB 222200 that have been previously reported (Sarau *et al*, 2000).

Adult male CD-1 mice were placed into activity monitors to acclimate and were injected with either vehicle or the NK-3 receptor antagonist SB 222200 (2.5, 5 mg/kg, s.c.). Thirty minutes later they were injected with cocaine (20 mg/kg, i.p.), and behavioral responses were monitored for an additional 60 mins. Behavioral activity was measured using the Digiscan DMicro System (Accusan, Columbus, OH, USA) that consisted of clean clear 20 x 20 x 42 cm plastic cages lined with horizontal photo-beams and detectors connected to an output computer. Activity was recorded as number of photo-beam breaks

as the animal moved about the cage. Ambulatory activity and stereotypic activity data were obtained from recorded number of consecutive and repetitive beam breaks, respectively.

To examine effects of repeated NK-3 receptor blockade on behavioral response to cocaine, mice were injected once daily with either vehicle or SB 222200 (5 mg/kg, s.c.) for 5 days (Days 1-5), followed by a 7-day drug-free period. On day 13, they were challenged with either saline, cocaine (20 mg/kg, i.p.), or SKF 82958 (0.125, 0.25 mg/kg, i.p.), and behavioral activity was measured. Another group of mice injected with either vehicle or SB 222200 were euthanized on day 13, and their brains were harvested for measurement of dopamine D1 receptor density by [^{3}H] SCH 23390 homogenate receptor binding as described below.

Dopamine D1 receptor homogenate binding

Striata were rapidly dissected on ice, pooled from 4 mice, and homogenized in 50 mM Tris HCl (pH 7.4 at 4^{o}C) with a polytron. Homogenates were centrifuged at 30,000 x g for 15 mins at 4^{o}C, pellets were resuspended in fresh cold Tris HCl, and were centrifuged again. The pellets were resuspended and incubated in a 37^{o}C shaking water bath for 30 mins. The homogenates were centrifuged, and resuspended in 50 mM Tris HCl, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$ at pH 7.4 at room temperature for the binding assays. Striatal membranes were incubated at room temperature for 45 mins with [^{3}H] SCH 23390 (0.1- 8 nM) and 1 μM mianserin to block binding to 5-HT receptors. Non-specific binding was determined from membranes incubated with [^{3}H] SCH 23390 and mianserin in the presence of 10 μM fluphenazine.

After incubation, membranes were harvested onto Whatman GF/B filter paper with cold Tris salt buffer using a Brandel cell harvester. Filter papers were transferred into vials filled with scintillation fluid (Cytoscint, Fisher Scientific) and radioactivity was counted by liquid scintillation. Protein concentrations of striatal homogenates were determined by the Lowry protocol (Lowry *et al*, 1951).

Data analysis

Acute behavioral data were analyzed by either one sample t-test or one-way ANOVA with Bonferroni post hoc tests using GraphPad Prism. Data from studies on behavioral responses to cocaine and the dopamine D1 receptor agonist SKF 82958 after repeated SB 222200 pretreatment were analyzed by two-way ANOVA with factors of Pretreatment (vehicle, SB 222200) and Challenge (saline, cocaine, SKF 82958), and further analyses were conducted using Bonferroni post hoc tests. Data from the [^{3}H] SCH 23390 receptor binding studies were analyzed by Scatchard analysis using GraphPad Prism, followed by an unpaired t-test. Statistical significance was determined at the alpha level of 0.05.

Results

Effect of acute administration of the NK-3 receptor antagonist SB 222200 on behavioral responses to cocaine

Ambulatory and stereotypic responses to cocaine in mice pretreated acutely with either vehicle or SB 222200 were measured. One-way ANOVA of ambulatory activity revealed a significant difference between the treatment groups ($F(3,50)=11.14$, $p< 0.0001$, Figure 2.1a). Bonferroni post hoc comparisons showed that cocaine significantly

increased ambulatory activity in vehicle pretreated animals ($p < 0.001$, vehicle-saline vs. vehicle-cocaine groups). There was no effect of SB 222200 on ambulatory activity induced by cocaine ($p > 0.05$, vehicle-cocaine vs. SB-cocaine groups). Statistical analysis of stereotypic activity revealed significantly different behavioral responses between the treatment groups ($F(3,50) = 13.13$, $p < 0.0001$, Figure 2.2b). Post hoc comparisons showed a significant stereotypic response to cocaine in vehicle pretreated mice ($p < 0.001$, vehicle-saline vs. vehicle-cocaine groups). Stereotypic activity induced by cocaine was attenuated by SB 222200 with significance at the 5 mg/kg dose ($p < 0.05$, vehicle-cocaine vs. 5SB-cocaine groups). SB 222200 pretreatment by itself had no significant effects on either ambulatory or stereotypic activity ($p > 0.05$, vehicle-saline vs. 5SB-saline groups).

Effect of prior administration of SB 222200 on subsequent behavioral hyperactivity induced by cocaine

Mice were injected once daily with either vehicle or SB 222200 for five days. After a 7-day drug-free period they were challenged with either saline or cocaine, and behavioral activity was measured. Statistical analysis of ambulatory activity (Figure 2.2a) showed a significant main effect of Challenge ($F(1,29) = 142.6$, $p < 0.0001$), but there was no effect of Pretreatment ($F(1,29) = 0.49$, $p > 0.05$) nor significant Pretreatment x Challenge interaction ($F(1,29) = 2.23$, $p > 0.05$). Cocaine significantly increased ambulatory activity (vehicle-saline vs. vehicle-cocaine groups) however pretreatment with SB 222200 for 5 days had no significant effect on ambulatory response to cocaine (vehicle-cocaine vs. 5SB-cocaine groups). Statistical analysis of stereotypic activity (Figure 2.2b) revealed a significant main effect of Challenge ($F(1,29) = 155.7$, $p < 0.0001$)

and Pretreatment ($F(1,29)= 4.88$, $p<0.05$,) as well as a significant Pretreatment x Challenge interaction ($F(1,29)= 4.71$, $p<0.05$). Bonferroni post hoc tests showed that cocaine induced a significant increase in stereotypic activity ($p<0.001$, vehicle-saline vs. vehicle-cocaine groups). Furthermore, prior repeated administration of SB 222200 resulted in a significantly higher stereotypic response to cocaine as compared to the cocaine response after vehicle pretreatment with a percent change of 34.7% ($p<0.01$, vehicle-cocaine vs. SB-cocaine groups). These data show that cocaine-induced stereotypic behavior was enhanced after prior administration of the NK-3 receptor antagonist SB 222200. Repeated SB 222200 administration did not significantly alter either basal ambulatory or stereotypic activity ($p>0.05$, vehicle-saline vs. SB-saline groups).

Effect of prior administration of the NK-3 receptor antagonist SB 222200 on dopamine D1 receptor-mediated behavioral activity

In order to study involvement of dopamine D1 receptors in the enhanced cocaine behavioral response after repeated NK-3 blockade, we examined changes in SKF 82958-mediated hyperactivity after repeated SB 222200 administration. Similar to the behavioral studies with cocaine, vehicle or SB 222200 was administered once daily for 5 days, and after a 7-day drug-free period, animals were challenged with either saline or the dopamine D1 receptor agonist SKF 82958, and behavioral responses were measured. Analysis of ambulatory activity (Figure 2.3a) showed a significant main effect of Challenge ($F (2,28) = 15.63$, $p< 0.0001$) but no effect of Pretreatment ($F (1,28) = 0.093$, $p> 0.05$), nor a Pretreatment x Challenge interaction ($F (2,28) = 0.46$, $p> 0.05$).

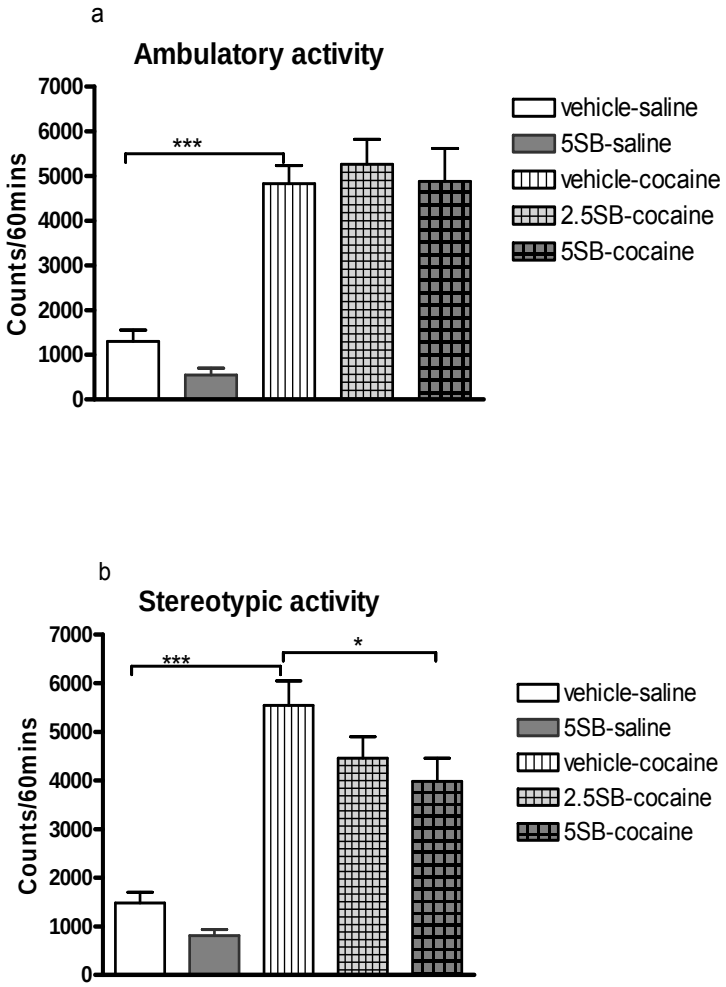

Figure 2.1. Effect of acute NK-3 receptor blockade on cocaine-induced ambulatory (a) and stereotypic (b) activity. Adult male CD-1 mice were injected with either vehicle or the NK-3 receptor antagonist SB 222200 (2.5, 5 mg/kg s.c.) 30 mins prior to either saline or cocaine (20 mg/kg i.p.). Within vehicle pre-treated groups, there was a significant increase in both ambulatory and stereotypic activity following cocaine. Pretreatment with SB 222200 significantly attenuated stereotypic activity in response to cocaine. Data are presented as mean ± SEM; N=6-19 mice/group (* $p<0.05$, *** $p< 0.001$).

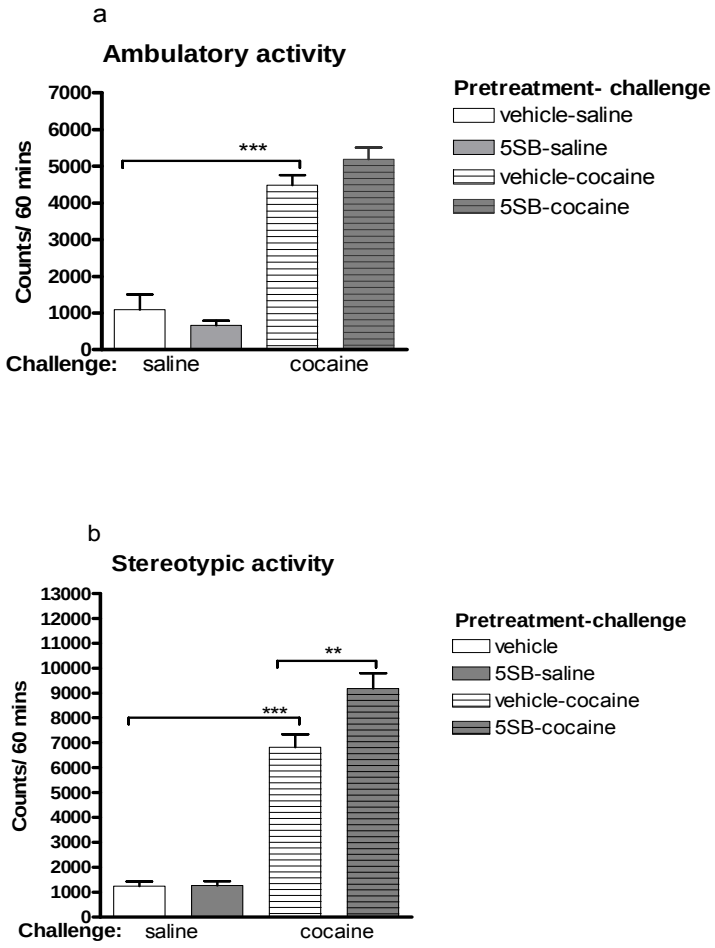

Figure 2.2. Effect of prior NK-3 receptor blockade on ambulatory (a) and stereotypic (b) activity of mice after a cocaine challenge. Adult male CD-1 mice were injected once daily with either vehicle or the NK-3 receptor antagonist SB 222200 (5 mg/kg, s.c.) for five days, and on day 13 challenged with either saline or cocaine (20 mg/kg. i.p.). Within vehicle pre-treated groups, cocaine increased both ambulatory and stereotypic activity. SB 222200 pretreatment significantly enhanced the behavioral response to cocaine in stereotypic activity (b) compared to vehicle. Data are presented as mean ± SEM; N=6-12 mice/group (* $p<0.05$, ** $p<0.01$, ***$p< 0.001$).

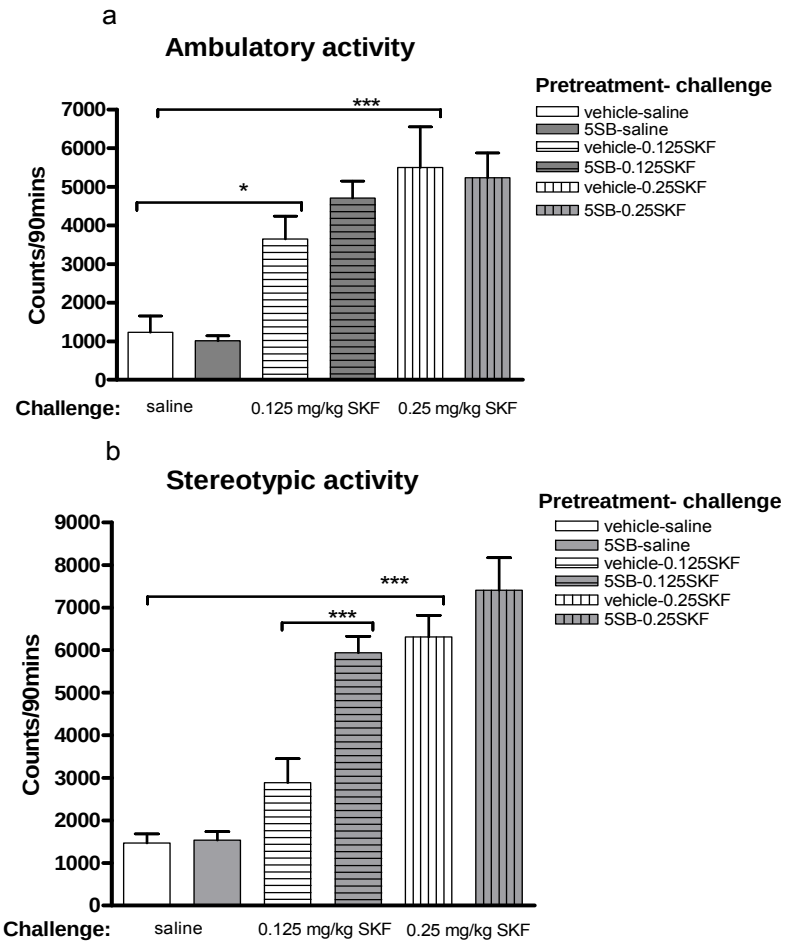

Figure 2.3. Effect of prior NK-3 receptor blockade on ambulatory (a) and stereotypic (b) activity of mice after a challenge with the dopamine D1 receptor agonist SKF 82958. Adult male CD-1 mice were injected once daily with either vehicle or the NK-3 receptor antagonist SB 222200 (5 mg/kg, s.c.) for five days, and on day 13 challenged with either saline or SKF 82958 (0.125 or 0.25 mg/kg, i.p.). Vehicle pre-treated animals had a significant behavioral response to SKF 82958 in ambulatory activity (a) and in stereotypic activity (b) at the 0.25 mg/kg dose. Pretreatment with SB 222200 significantly enhanced stereotypic activity following a challenge injection of 0.125 mg/kg SKF 82958. Data are presented as mean ± SEM; N=5-6 mice/group (* $p<0.05$, ** $p<0.01$, *** $p< 0.001$).

Both doses of SKF 82958 induced a significant increase in ambulatory activity, however ambulatory activity was not altered by SB 222200. Statistical analysis of stereotypic activity (Figure 2.3b) revealed a significant main effect of Challenge ($F(2,28) = 67.43$, $p < 0.0001$) and Pretreatment ($F(1,28) = 13.74$, $p < 0.001$) as well as a significant Pretreatment x Challenge interaction ($F(2,28) = 5.41$, $p < 0.01$). Bonferroni post hoc tests showed that 0.25 mg/kg SKF 82598 induced a significant increase in stereotypic activity in vehicle pretreated animals ($p<0.001$, vehicle-saline vs. vehicle-0.25SKF groups), although the lower dose of SKF 82958 (0.125 mg/kg) did not significantly alter stereotypic activity ($p>0.05$, vehicle-saline vs. vehicle-0.125SKF groups). Stereotypic activity following 0.125 mg/kg SKF 82958 was significantly higher in mice pretreated with SB 222200 compared to those pretreated with vehicle ($p<0.001$, vehicle-0.125SKF vs. SB-0.125SKF groups). These data demonstrate that prior administration of the NK-3 receptor antagonist SB 222200 enhanced stereotypic behavior to a sub-effective dose of the dopamine D1 agonist SKF 82958. Repeated SB 222200 administration did not significantly alter either basal ambulatory or stereotypic activity ($p>0.05$, vehicle-saline vs. SB-saline groups).

Dopamine D1 receptor up-regulation in the striatum of mice pretreated with the NK-3 receptor antagonist SB 222200

Since both behavioral responses to cocaine and SKF 82958 were enhanced following SB 222200 administration, the effect of SB 222200 on dopamine D1 receptor density was assessed. Mice were administered either vehicle or SB 222200 for 5 days and left drug-free for 7 days. On day 13, the striatum of animals was harvested to study

changes in dopamine D1 receptor density by [^{3}H] SCH 23390 homogenate binding. Results from Scatchard analyses showed a significant increase in B_{max} of striatal membranes from SB 222200 injected mice as compared to vehicle injected mice (unpaired t- test; $t(6)= 2.537$, $p<0.05$). B_{max} values of dopamine D1 receptor binding of striatal membranes from the SB treatment group were 19.7% higher than controls (Table 2.1, Figure 2.4) indicating an increase in dopamine D1 receptor density in the striatum. There was no significant change in K_D values after SB 222200 administration (Table 2.1).

	B_{max}	K_D
	fmol/mg protein	nM
Vehicle	742.5 ± 48.6	2.6 ± 0.3
SB 222200	888.5 ± 104.6*	2.3 ± 0.3
% change	+19.7	

Table 2.1. B_{max} and K_D values from Scatchard analyses of [^{3}H] SCH 23390 homogenate binding assays. Adult male CD-1 mice were injected once daily with either vehicle or the NK-3 receptor antagonist SB 222200 (5 mg/kg, s.c.) for five days. After a 7-day drug-free period, dopamine D1 receptor density in the striatum was measured using [^{3}H] SCH 23390. Scatchard analyses showed an up-regulation of dopamine D1 receptors in the striatum of animals administered SB 222200 compared to animals injected with vehicle. Data are presented as mean ± SD; N=4 separate assays (* $p<0.05$ vehicle vs. SB 222200).

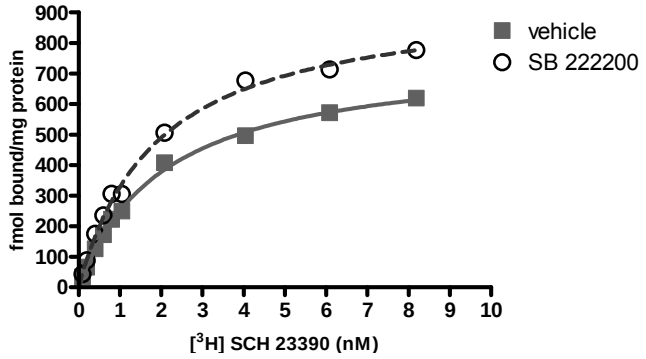

Figure 2.4. [^{3}H] SCH 23390 binding to striatal membranes from mice injected with vehicle and SB 222200. Adult male CD-1 mice were injected once daily with either vehicle or the NK-3 receptor antagonist SB 222200 (5 mg/kg, s.c.) for five days, and on day 13 dopamine D1 receptor densities in striatal membranes were measured using [^{3}H] SCH 23390. Dopamine D1 receptor density was higher in striatal membranes from animals treated with SB 222200 compared to membranes from vehicle treated animals. A representative saturation binding curve is shown.

Discussion

Modulation of NK-3 receptor activity has been shown to alter dopamine-mediated behaviors. Previous studies have reported that administration of NK-3 receptor agonists elicits dopamine-mediated behaviors which include hyper-locomotion and stereotypy (Deschamps *et al*, 2005; Elliott *et al*, 1991; Stoessl *et al*, 1991). The NK-3 receptor antagonist SR 142801 attenuates locomotion and stereotypic activity induced by the dopamine D1 receptor agonist SKF 82958 (Bishop *et al*, 2004) or cocaine in rats (Jocham *et al*, 2006) and non-human primates (De Souza Silva *et al*, 2006b), however by itself has no effect on basal dopamine levels (Marco *et al*, 1998) or basal behavioral activity (Bishop *et al*, 2004; De Souza Silva *et al*, 2006b; Jocham *et al*, 2006). In support of these findings, our study shows that acute blockade of NK-3 receptors by SB 222200 attenuated behavioral hyperactivity induced by cocaine, particularly stereotypic activity. In addition, acute administration of SB 222200 did not significantly alter basal behavioral activity.

One potential mechanism by which NK-3 receptors could modulate dopamine-mediated behaviors is through its effects on dopaminergic neuronal activity in the SN and VTA (Figure 2.5). Previous studies have shown the NK-3 receptor agonist senktide when administered into the substantia nigra and VTA of anesthetized rats (Overton *et al*, 1992) or applied locally on midbrain slices (Keegan *et al*, 1992) increases the firing rate of dopaminergic neurons. Further, activation of NK-3 receptors in the substantia nigra and VTA increases dopamine release and DOPAC tissue content in the striatum, nucleus accumbens and prefrontal cortex (Bannon *et al*, 1995; Humpel *et al*, 1991; Marco *et al*, 1998).

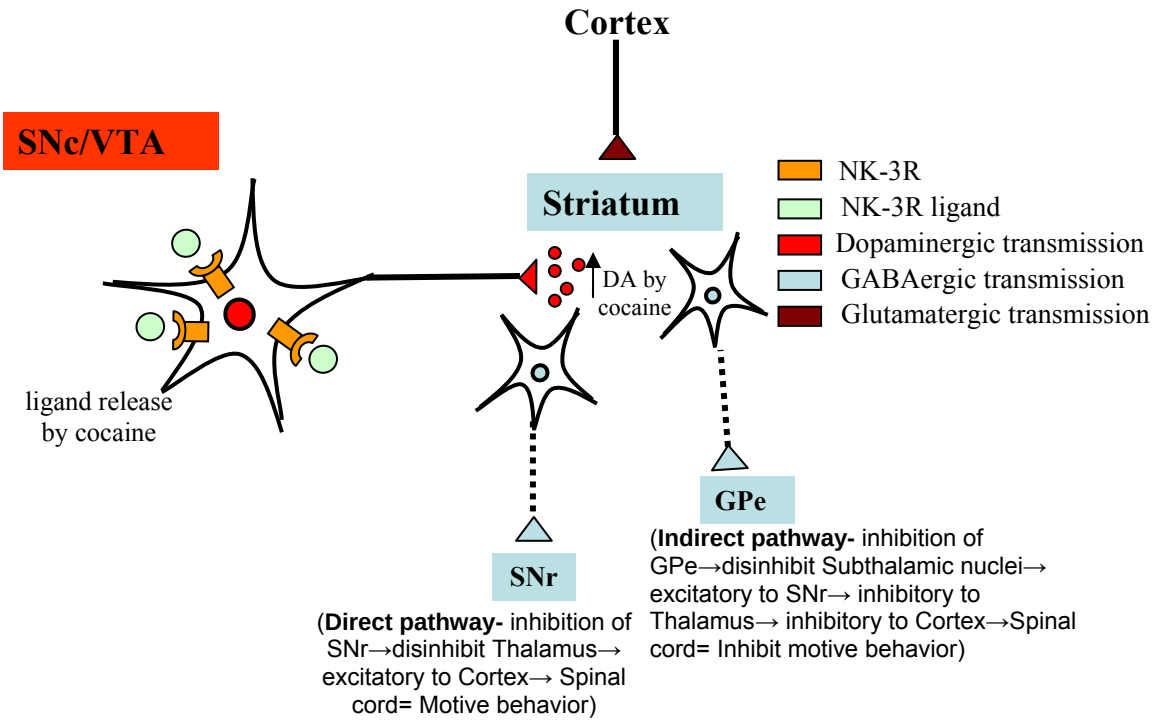

Figure 2.5. A proposed mechanism of modulation of cocaine-induced hyperactivity by NK-3 receptors. Cocaine increases extracellular dopamine levels in the striatum and presumably activates NK-3 receptors via the release of endogenous tachykinins. Activation of NK-3 receptors in the SNc/VTA facilitates dopaminergic neurotransmission which modulates the direct/indirect pathways, resulting in behavioral hyperactivity.

In the present study, acute administration of the NK-3 receptor antagonist SB 222200 attenuated hyperactivity induced by cocaine, which may point to indirect activation of NK-3 receptors in the substantia nigra and VTA by cocaine. Therefore, we propose that the inhibition of pre-synaptic uptake of dopamine in the striatum and activation of NK-3 receptors in the substantia nigra and VTA by cocaine may in part mediate behavioral hyperactivity (Figure 2.5). Since NK-3 receptors in the substantia nigra and VTA can modulate dopaminergic function, it is likely that this mechanism is involved in cocaine-induced behaviors.

Another mechanism by which NK-3 receptors could regulate dopaminergic behaviors is by modulating the activity of striatal cholinergic interneurons. Cholinergic interneurons in the striatum have been shown to alter the excitability of GABAergic medium spiny neurons and thereby facilitate neurotransmission from the cortex or thalamus to regulate dopamine-mediated behaviors (Kawaguchi *et al*, 1995; Preston *et al*, 2000; Saka *et al*, 2002). Application of NK-3 receptor agonists causes a Ca^{2+}-dependent depolarization of striatal cholinergic interneurons (Preston *et al*, 2000) and a phospholipase C and protein kinase C-dependent release of acetylcholine in striatal slices (Arenas *et al*, 1991; Preston *et al*, 2000).

Long-term effects of prior NK-3 receptor blockade on dopaminergic hyperactivity have not been reported to date. Our findings demonstrate that prior repeated NK-3 receptor blockade by SB 222200 enhanced stereotypic hyperactivity induced by a cocaine challenge. Since cocaine inhibits the re-uptake of dopamine, norepinephrine and serotonin, we also investigated effects of a selective dopamine D1 receptor agonist SKF 82958. Behavioral hyperactivity induced by SKF 82958 was enhanced after prior

repeated NK-3 receptor blockade similar to our findings with cocaine. The observed changes in behavioral hyperactivity were accompanied by dopamine D1 receptor up-regulation in the striatum after prior NK-3 receptor blockade suggesting a possible mechanism for the augmented behavioral response to cocaine and SKF 82958. Collectively, these findings suggest that in addition to acutely modulating dopamine, NK-3 receptor blockade can produce long-term changes in dopamine-mediated behaviors and receptor expression.

The subsequent behavioral super-sensitivity of dopamine receptors caused by repeated administration of NK-3 receptor antagonist SB 222200 was shown in the present study to be associated with increased density of dopamine D1 receptors in the striatum. We propose that the change in behaviors may be an outcome of prolonged depression of dopamine transmission in the striatum. Previous studies have shown that 6-OHDA lesions of dopaminergic terminals results in enhancement of dopamine-mediated behaviors (Breese *et al*, 1987). Likewise, chronic administration of dopamine receptor antagonists results in dopamine receptor super-sensitivity, and subsequent enhancement of behaviors which are accompanied by dopamine receptor up-regulation (Hess *et al*, 1986; Hess *et al*, 1988). In addition, chronic administration of dopamine receptor antagonists enhances hyperactivity induced by cocaine (Mattingly *et al*, 1996). Since acute blockade of NK-3 receptors attenuates dopaminergic transmission, prolonged blockade of NK-3 receptors might eventually lead to dopamine receptor super-sensitivity. In this study, the enhancement of subsequent behavioral responses to both cocaine and the dopamine D1 receptor agonist SKF 82958 and the increase in dopamine D1 receptor density in the striatum is suggestive of dopamine receptor super-sensitivity after repeated

NK-3 receptor blockade. Of particular interest, recent findings by Nordquist and colleagues demonstrate enhanced behavioral responses to amphetamine in mice with genetic deletion of the NK-3 receptor, and also changes in dopamine D1 receptor binding in the striatum (Nordquist *et al*, 2008). However, unlike Nordquist and colleagues who report a slight decrease in striatal dopamine D1 receptors, we found increased dopamine D1 receptor binding in the striatum in mice 7 days after repeated NK-3 receptor blockade. Collectively, these findings point to compensatory changes of striatal dopamine D1 receptors in the NK-3 receptor knockout mouse and in a mouse 7 days drug-free after prior repeated NK-3 receptor blockade, although these changes may not be comparable. In conclusion, our study demonstrates long-term change in dopamine-mediated behaviors after prior blockade of NK-3 receptors and dopamine receptor up-regulation that is possibly an outcome of prolonged depression of dopaminergic transmission.

Investigations on NK-3 receptor function in the central nervous system are currently being conducted in various animal models that include mice, rats, gerbils, guinea pigs and non-human primates. There is evidence of differences in NK-3 receptor structure, expression in the central nervous system, and ligand selectivity among these species and also in humans (Buell *et al*, 1992; Langlois *et al*, 2001; Maggi, 1995; Mileusnic *et al*, 1999), which has presented some obstacles in the study of mammalian NK-3 receptors. For instance, in comparison of the rat, gerbil and guinea pig, only the guinea pig has no NK-3 receptor binding in the substantia nigra par compacta and the ventral tegmental area, and only the rat has NK-3 receptor binding in the anterior caudate putamen (Langlois *et al*, 2001). In addition, indication of species differences in pharmacological characteristics of NK-3 receptor antagonists further complicate

investigations in these animal models. For instance, the NK-3 receptor antagonist SR 1421801 shows the most affinity in binding in the guinea pig with a Ki value of 0.11 nM as compared to the rat (15 nM), gerbil (0.42 nM) and human (0.21 nM) NK-3 receptors (Emonds-Alt *et al*, 1995). The NK-3 receptor antagonist used in this study, SB 222200, also shows some species differences in ligand binding with a Ki value of 4.4 nM in the human NK-3 receptor, 3 nM in the guinea pig, 88 nM in the rat and 174 nM in the mouse (Sarau *et al*, 2000). However, SB 222200 has been shown to be efficacious in inhibiting NK-3 receptor mediated Ca^{2+} mobilization and behavioral responses in a concentration dependent manner in mice (Sarau *et al*, 2001; Sarau *et al*, 2000). In addition, studies have shown there are differences in selectivity of NK-3 receptor for the endogenous ligands NK-B and substance P. In displacement radioligand binding studies, the rat NK-3 receptor binds 452-fold for NK-B over substance P (Shigemoto *et al*, 1990), but in contrast the human NK-3 receptor selectivity for NK-B over substance P is much less, by about 97-fold (Buell *et al*, 1992). The same observation is also noted with comparisons between the mouse and human NK-3 receptor with the mouse NK-3 receptor selectivity of NK-B over substance P being about 2140-fold selective and the human NK-3 receptor much less by about 860-fold (Sarau *et al*, 2001). These species differences present some obstacles in the study of NK-3 receptors as a novel therapeutic target for neuropsychiatric disorders in animal models. Given the presented limitations in using a mouse model, our findings still offer insight into function of NK-3 receptors and possible long-term adaptations in dopaminergic behaviors as a result of repeated NK-3 receptor blockade.

NK-3 receptors are being studied as potential therapeutic targets for treatment of various human pathologies. In the periphery, NK-3 receptors can be found mainly on

nerve endings of c-fibers of primary afferent neurons innervating the respiratory, gastrointestinal and urinary tracts (Patacchini *et al*, 2001). In these areas, NK-3 receptors play a role in pathological inflammatory processes implicated in diseases such as asthma, inflammatory bowel syndrome and cystitis (Canning, 2006; Patacchini *et al*, 2001). In the central nervous system, their role in modulating dopamine transmission makes them potential targets in treatment of neuropsychiatric illnesses including schizophrenia and various affective disorders, and also Parkinson's disease (Panocka *et al*, 2001; Ribeiro *et al*, 1998; Spooren *et al*, 2005). Findings from the present study lead us to propose further inquiry into possible consequences of chronic use of agents that function as NK-3 receptor antagonists. Their long-term use may cause dopamine receptor supersensitivity, similar to what is observed with use of classical antipsychotics that cause tardive syndromes in patients.

In summary, our findings show that acute blockade of NK-3 receptors attenuated but repeated blockade enhanced subsequent dopamine-mediated behaviors, and concurrently up-regulated dopamine D1 receptors in the striatum. These findings point to a potential role of NK-3 receptors in mediating acute and long-term changes in dopaminergic transmission and indicate that there is a functional interaction between NK-3 and dopamine transmission in the striatum.

CHAPTER 3

A ROLE OF NK-3 RECEPTORS IN THE DEVELOPMENT AND EXPRESSION OF COCAINE BEHAVIORAL SENSITIZATION

Introduction

Cocaine causes its psychomotor as well as its reinforcing effects by inhibiting re-uptake of dopamine and to a lesser extent norepinephrine and serotonin into pre-synaptic terminals, causing elevated levels of these neurotransmitters in the synapse (Reith, 1986; Ritz *et al*, 1987). Upon repeated intermittent exposure, cocaine induces behavioral sensitization that is characterized as a progressively heightened behavioral response after drug re-exposure (Post *et al*, 1976), which persists after long periods of drug abstinence (Heidbreder *et al*, 1996). Behavioral sensitization may be relevant to the etiology of other conditions such as drug-induced psychoses (Bartlett *et al*, 1997; Brady *et al*, 1991; Satel and Edell, 1991) and schizophrenia (Glenthoj and Hemmingsen, 1997; Laruelle, 2000; Lieberman *et al*, 1997).

Neurokinin-B, substance P and neurokinin-A are members of the mammalian tachykinin neuropeptides with the common carboxyl terminus sequence –Phe-X-Gly-Leu-Met-NH_2. Displacement binding studies indicate that neurokinin-B is the likely endogenous ligand for NK-3 receptors (Shigemoto *et al*, 1990), however neurokinin-A and substance P can also activate NK-3 receptors. Neurokinin-B is derived by alternate splicing and post-translational processing from preprotachykinin B precursor peptide and substance P and neurokinin-A are both derived from preprotachykinin A (Massi *et al*, 2000). Activation of NK-3 receptors causes the generation of the second messengers 1,4,5 inositol triphosphate (IP_3) and diacylglycerol through a Gq protein-dependent

activation of phospholipase C (Khawaja *et al*, 1996; Maggi, 1995), which results in Ca^{2+} mobilization and induction of Ca^{2+}-dependent signaling pathways.

NK-3 receptors located in the substantia nigra, ventral tegmental area (VTA) and prefrontal cortex are thought to regulate dopaminergic neurotransmission and locomotive behaviors. Activation of NK-3 receptors in the substantia nigra and VTA stimulates dopaminergic neuronal activity (Keegan *et al*, 1992; Overton *et al*, 1992) and increases dopamine release and metabolism in the nucleus accumbens, dorsal striatum, and prefrontal cortex (Bannon *et al*, 1995; Humpel *et al*, 1991; Marco *et al*, 1998). Behaviors induced by cocaine and dopamine receptor agonists are attenuated by acute administration of NK-3 receptor antagonists (Bishop *et al*, 2004; De Souza Silva *et al*, 2006b; Jocham *et al*, 2006). In addition, NK-3 receptor agonists have been shown to potentiate cocaine-induced behaviors (de Souza Silva *et al*, 2006a; Jocham *et al*, 2007). These findings demonstrate that NK-3 receptors can modulate dopaminergic behavior.

The purpose of the present study was to determine the role of NK-3 receptors in the development and expression of behavioral sensitization to cocaine. Since previous studies have shown that NK-3 receptor blockade attenuates dopamine-mediated behaviors and also cocaine's acute behavioral effects, we hypothesized that cocaine-induced activation of NK-3 receptors is necessary for the development and expression of behavioral sensitization.

Materials and methods

Animals

Adult male CD-1 mice (Charles River Laboratories, Raleigh, NC, USA) were group housed (4-6 per cage) in a temperature and humidity controlled environment on a

12-h light–dark cycle (lights on at 7AM) with *ad libitum* access to food and water. Animals were handled daily prior to the beginning of the study. All experiments were conducted in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals and with approval from Temple University Institutional Animal Care and Use Committee.

Drugs

Cocaine hydrochloride was generously provided by the National Institute on Drug Abuse and dissolved in sterile 0.9% saline. (*S*)-3-methyl-2-phenyl-N-(1-phenylpropyl)-4-quinolinecarboxamide (SB 222200) was obtained from Sigma Aldrich and dissolved in a vehicle composed of 60% polyethylene glycol (PEG-200) and 40% distilled water. Cocaine was injected intraperitoneally in a volume of 3 ml/kg body weight, and SB 222200 was injected subcutaneously in a volume of 2 ml/kg.

Assessment of behavioral sensitization

Behavioral activity was measured using the Digiscan DMicro System (Accusan, Columbus, OH, USA), which consists of clean clear 20 x 20 x 42 cm plastic cages lined with horizontal photo-beams and detectors that were interfaced with an output computer. Ambulatory activity was recorded as counts of consecutive photo beam breaks.

In order to examine effects of the NK-3 receptor antagonist SB 222200 on the development of behavioral sensitization to cocaine, adult male CD-1 mice were administered with either vehicle or the NK-3 receptor antagonist SB 222200 (2.5 or 5 mg/kg, s.c.) followed 30 mins later by either saline or cocaine (20 mg/kg, i.p.) once daily for 5 days (days 1-5). Mice were left untreated for 7 days, and on day 13 were challenged

with cocaine (20 mg/kg, i.p.) in the absence of SB 222200, and ambulatory activity was measured for 60 mins.

To examine effects of the NK-3 receptor antagonist SB 222200 on the expression of behavioral sensitization to cocaine, mice were injected daily with either saline or cocaine (20 mg/kg, i.p.) for 5 days (days 1-5), followed by a 7-day drug-free period. On day 13, mice were injected with either vehicle or SB 222200 (5 mg/kg, s.c.) and 30 mins later were challenged with an injection of cocaine (20 mg/kg, i.p.), and ambulatory activity was measured for 60 mins.

Data analysis

Data were analyzed by one-way ANOVA with Bonferroni post hoc tests (GraphPad Prism). Statistical significance was determined at the alpha level of 0.05.

Results

Pretreatment with the NK-3 receptor antagonist SB 222200 prevents the development of behavioral sensitization to cocaine

Ambulatory responses to a cocaine challenge were measured in mice pretreated with either vehicle or SB 222200 and saline or cocaine for 5 days (Figure 3.1a, 3.1b). Statistical analyses of total ambulatory activity revealed a significant difference between treatment groups ($F(4,39)=3.93$, $p< 0.01$, Figure 3.1b). Bonferroni post hoc comparisons showed that a cocaine challenge induced significantly higher ambulatory activity in mice administered repeated cocaine than in mice given saline ($p <0.05$, vehicle-saline vs. vehicle-cocaine groups). Mice administered SB 222200 prior to repeated cocaine for 5

days did not show a sensitized response to a subsequent cocaine challenge ($p<0.01$, vehicle-cocaine vs. 5SB-cocaine groups), demonstrating that administration of SB 222200 blocked the development of cocaine-induced behavioral sensitization.

Administration of the NK-3 receptor antagonist SB 222200 blocks the expression of behavioral sensitization to cocaine

Ambulatory responses were measured in mice pretreated with SB 222200 30 mins prior to a cocaine challenge after previous administration of repeated saline or cocaine (Figure 3.2a, 3.2b). Statistical analyses of ambulatory activity revealed a significant difference between treatment groups ($F(3,38)=2.87$, $p< 0.05$, Figure 3.2b). Bonferroni post hoc comparisons showed that mice administered repeated cocaine had significantly higher ambulatory responses to a subsequent cocaine challenge than mice given repeated saline ($p <0.05$, vehicle-saline vs. vehicle-cocaine groups) indicative of sensitization. However, the sensitized response was blocked by administration of 5 mg/kg SB 222200 30 mins prior to the cocaine challenge ($p>0.05$, cocaine-vehicle vs. cocaine-5SB groups). These data show that pretreatment with SB 222200 during a cocaine challenge blocked the expression of cocaine-induced behavioral sensitization.

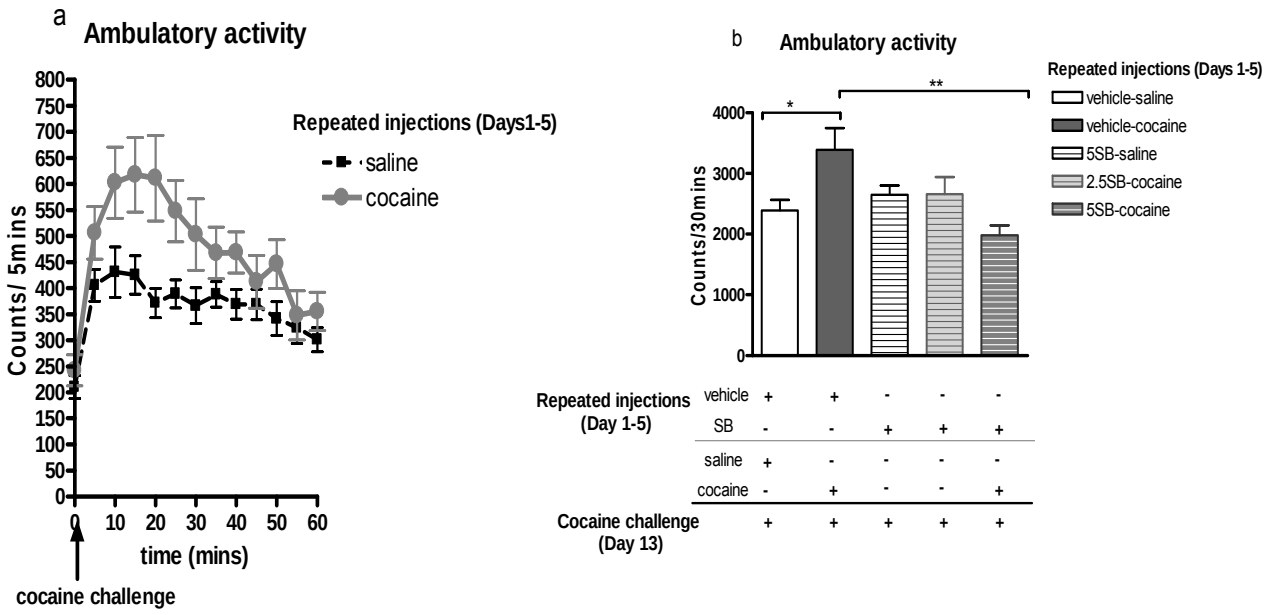

Figure 3.1. Effect of NK-3 receptor blockade on the development of behavioral sensitization to cocaine; time course data (a) and cumulative data (b). Adult male CD-1 mice were injected with either vehicle or the NK-3 receptor antagonist SB 222200 (2.5 or 5 mg/kg s.c.) 30 mins prior to a cocaine (20 mg/kg, i.p.) injection for 5 days. After a seven day drug-free period, all mice were challenged with cocaine (20 mg/kg, i.p.) and behavioral responses were measured. Mice injected repeatedly with cocaine had significantly increased ambulatory activity compared to saline animals (a, b) indicating a sensitized response. Administration of SB 222200 prior to cocaine blocked the sensitized behavioral response to a cocaine challenge in mice given repeated cocaine. Data are presented as mean ± SEM; N=7-12 mice/group (* $p<0.05$, ** $p< 0.01$).

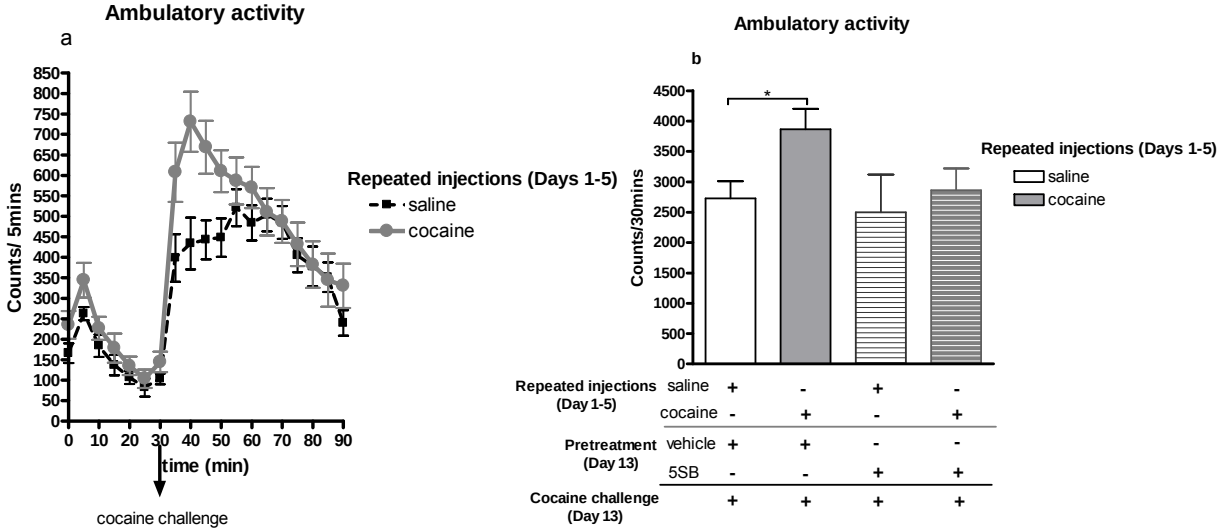

Figure 3.2. Effect of NK-3 receptor blockade on the expression of behavioral sensitization to cocaine; time course data (a) and cumulative data (b). Adult male CD-1 mice were injected once daily with either saline or cocaine for five days. After a seven day drug-free period, they were administered either vehicle or the NK-3 receptor antagonist SB 222200 (5 mg/kg, s.c.) 30 mins before being challenged with cocaine (20 mg/kg, i.p.). Mice injected repeatedly with cocaine had significantly increased ambulatory activity compared to saline mice (b) indicating a sensitized response. SB 222200 administration 30 mins prior to a cocaine challenge did not significantly alter ambulatory activity in saline control animals, but blocked the enhanced behavioral response to cocaine in animals given repeated cocaine. Data are presented as mean ± SEM; N=7-14 mice/group (* $p<0.05$).

Discussion

A role of NK-3 receptors in regulating cocaine-induced behavioral responses has been demonstrated in studies showing acute administration of NK-3 receptor agonists enhance and NK-3 receptor antagonists administration attenuates cocaine-induced behaviors (de Souza Silva *et al*, 2006a, b; Jocham *et al*, 2007; Jocham *et al*, 2006; Nwaneshiudu and Unterwald, 2009)as summarized in the previous chapter. The present study examined potential NK-3 receptor regulation of behavioral sensitization to cocaine. Our findings show that administration of the NK-3 receptor antagonist SB 222200 prior to repeated cocaine blocked the development of sensitized behavioral response after a subsequent cocaine challenge. SB 222200 administration before a cocaine challenge prevented the expression of behavioral sensitization. These findings suggest that cocaine-mediated activation of NK-3 receptors is important for the development and expression of behavioral sensitization.

Behavioral sensitization is thought to involve long-term molecular adaptations in neurotransmission in the mesolimbic dopamine pathway resulting from repeated intermittent drug administration (Anderson and Pierce, 2005; Pierce *et al*, 1997; Vanderschuren *et al*, 2000). Some of these adaptations have been previously described and include changes in dopaminergic neurotransmission in the nucleus accumbens. Studies have demonstrated an enhanced inhibition of nucleus accumbens neurons to locally applied dopamine (Beurrier and Malenka, 2002; Henry and White, 1991) that results from persistent dopamine D1 receptor super-sensitivity (Henry *et al*, 1991). In addition, there is also increased expression of dopamine D1 receptors, increased dopamine D1 receptor mediated activation of cAMP signaling and induction of gene

expression in the nucleus accumbens (Unterwald *et al*, 1996; Zhang *et al*, 2005). In the present study, we have demonstrated that antagonism of NK-3 receptors prevented behavioral sensitization to cocaine, which may be through NK-3 receptor mediated modulation of dopamine neurotransmission to the nucleus accumbens.

Effects of NK-3 receptor blockade during the development of behavioral sensitization to cocaine may also point to a mechanism involving NK-3 receptor activation by cocaine via released endogenous ligands. Brain regions involved in locomotive behaviors such as the caudate putamen, nucleus accumbens and cerebral cortex have been shown to express endogenous ligands for NK-3 receptors such as NK-B (Burgunder *et al*, 1989; Marksteiner *et al*, 1992c; Warden *et al*, 1988). Acute and repeated cocaine administration can increase preprotachykinin mRNA levels in the striatum (Adams *et al*, 2001; Mathieu-Kia and Besson, 1998). Elevation of this precursor molecule of the endogenous tachykinin peptides can increase the activation of NK-3 receptors. For that reason, activation of NK-3 receptors may play a role in behavioral sensitization to cocaine.

Behavioral sensitization is a model that is useful in studying the neuroadaptations that likely contribute to compulsive craving seen in cocaine-seeking behaviors (Robinson *et al*, 2001; Vanderschuren *et al*, 2000). This is based on the observations that the brain regions that are involved in drug-induced sensitization also mediate salient-motivation and drug reward. In addition to drug addiction, sensitization can also be used to dissect the possible etiologies of schizophrenia (Glenthoj *et al*, 1997; Laruelle, 2000; Lieberman *et al*, 1997). The concept of neurochemical sensitization of the mesolimbic dopamine pathway has been postulated to be a key step that is associated with the pathogenesis of

schizophrenia. Clinical studies show that schizophrenic patients have increased sensitivity to the effects of acute psychostimulant administration and their responses are similar in nature to the spontaneous psychosis during active episodes of schizophrenia (Laruelle, 2000). Sensitization to the psychosis-inducing effects of cocaine has also been reported by several groups (Bartlett *et al*, 1997; Brady *et al*, 1991; Satel *et al*, 1991). From these studies in conjunction with our findings, we postulate that the changes in the brain resulting in behavioral sensitization to cocaine may be similar in nature to those present in schizophrenia. In addition, NK-3 receptors may also play a contributory role in the etiology of schizophrenia.

In summary, the findings of this study demonstrate that NK-3 receptor antagonism blocks the development and expression of cocaine behavioral sensitization. These findings suggest that cocaine causes activation of NK-3 receptors, which mediates the development and expression of sensitization. There is possible NK-3 receptor involvement in the etiologies of cocaine addiction and schizophrenia. In future investigations, NK-3 receptors can be studied as putative therapeutic targets for treatment of these aberrant states.

CHAPTER 4

FUNCTION OF NK-3 RECEPTORS IN COCAINE-INDUCED CHANGES IN GSK3 PHOSPHORYLATION IN THE NUCLEUS ACCUMBENS

Introduction

Neurokinin-B, substance P and neurokinin-A are the mammalian tachykinin neuropeptides with the common carboxyl terminus sequence –Phe-X-Gly-Leu-Met-NH_2 that can activate NK-3 receptors under physiological conditions. Neurokinin-B is derived by alternate splicing and post-translational processing from preprotachykinin B and substance P and neurokinin-A both from preprotachykinin A (Massi *et al*, 2000). NK-3 receptor activation causes a Gq protein-dependent activation of phospholipase C (Khawaja *et al*, 1996; Maggi, 1995) and subsequent phosphoinositol 4, 5 biphosphate (PIP_2) breakdown into 1,4,5 inositol triphosphate (IP_3) and diacylglycerol, which results in elevated Ca^{2+} levels and Ca^{2+}dependent signaling cascades.

Glycogen synthase kinase 3 (GSK3) is expressed in the central nervous system in two isoforms, GSK3α and GSK3β, both of which are regulated by phosphorylation at serine 21 and serine 9 residues, respectively, leading to inhibition of kinase activity (Grimes *et al*, 2001b). Phosphorylation of GSK3 has been shown to be primarily regulated either by protein kinase B (Akt), DARPP-32, P70 S6 kinase, MAP kinase-activated protein kinase-1 (MAPKAP-1), or protein kinase A (Alessi *et al*, 1996; Li *et al*, 2000; Svenningsson *et al*, 2003). The activated form of GSK3 phosphorylates a variety of proteins including signaling molecules, structural proteins and transcription factors (Jope and Bijur, 2002). The role of GSK3β in regulating various cellular functions is well

established and includes gene expression, cell structure, neural plasticity, cell survival and apoptosis.

There is evidence that GSK3β activity modulates dopamine-mediated behaviors (Beaulieu *et al*, 2004; Miller *et al*, 2009; Prickaerts *et al*, 2006). In particular, administration of GSK3β inhibitors in dopamine transporter knockout mice attenuates hyperactivity in a novel environment (Beaulieu *et al*, 2004). GSK3β heterozygous mice show attenuated behavioral responses to amphetamine (Beaulieu *et al*, 2004). In addition, the GSK3β inhibitor SB 216763 attenuates cocaine-induced hyperactivity, the development of behavioral sensitization to cocaine and cocaine reward (Miller *et al*, 2009). Conversely, overexpression of an activated construct of GSK3β results in increased behavioral hyperactivity (Prickaerts *et al*, 2006). These studies demonstrate the significance of GSK3β in cocaine-induced behaviors.

NK-3 receptors have also been shown to regulate dopaminergic neurotransmission and behaviors presumably by modulating dopaminergic neuronal activity (Keegan *et al*, 1992; Overton *et al*, 1992) and dopamine release and metabolism in the nucleus accumbens, striatum, and prefrontal cortex (Bannon *et al*, 1995; Humpel *et al*, 1991; Marco *et al*, 1998). Also described in chapter 2 and in agreement with past studies, cocaine-induced behaviors are also modulated by acute administration of NK-3 receptor agonists and antagonists(Bishop *et al*, 2004; de Souza Silva *et al*, 2006a, b; Jocham *et al*, 2007; Jocham *et al*, 2006; Nwaneshiudu *et al*, 2009). In addition, our findings from the previous chapter implicate a role of NK-3 receptor activity in the development and expression of behavioral sensitization to cocaine.

The present study investigated the role of NK-3 receptors in the regulation of GSK3 phosphorylation in the nucleus accumbens by cocaine. In particular, experiments were designed to examine changes in phosphorylation of GSK3α and GSK3β during acute cocaine that causes behavioral hyperactivity, and after previous exposure to repeated cocaine that would cause sensitized behavioral responses. Collectively, since NK-3 receptor blockade attenuates the acute and sensitized behavioral effects of cocaine and GSK3 activity also regulates acute cocaine-induced hyperactivity and behavioral sensitization, we hypothesized that activity of NK-3 receptors can modulate phosphorylation of GSK3α and GSK3β in the nucleus accumbens induced by cocaine.

Materials and methods

Animals

Adult male CD-1 mice (Charles River Laboratories, Raleigh, NC, USA) were group-housed (4-6 per cage) in a temperature and humidity controlled environment on a 12-h light–dark cycle (lights on at 7AM) with *ad libitum* access to food and water. Animals were handled daily prior to the beginning of the study. All experiments were conducted in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals and with approval from Temple University Institutional Animal Care and Use Committee.

Drugs

Cocaine hydrochloride was generously provided by the National Institute on Drug Abuse and dissolved in a sterile 0.9% saline. (*S*)-3-methyl-2-phenyl-N-(1-phenylpropyl)-

4-quinolinecarboxamide (SB 222200) was obtained from Sigma Aldrich and dissolved in a vehicle composed of 60% polyethylene glycol (PEG-200) and 40% distilled water. Cocaine was injected intraperitoneally in a volume of 3 ml/kg body weight and SB 222200 was injected subcutaneously in a volume of 2 ml/kg.

Drug treatments

In an acute study, mice were administered either vehicle or SB 222200 (2.5 or 5 mg/kg, s.c.) followed 30 mins later by an injection of either saline or cocaine (20 mg/kg, i.p.). Mice were euthanized 20 mins later following 15-sec CO_2 exposure and decapitation. For another study that examined effects of repeated cocaine exposure, mice were injected once daily with either vehicle or SB 222200 (5 mg/kg, s.c.) followed 30 mins later by either saline or cocaine (20 mg/kg, i.p.) for 5 days (days 1-5), and left drug free for 7 days. On Day 13, mice were challenged with either saline or cocaine (20 mg/kg, i.p.) and euthanized 20 mins later by decapitation following 15-sec CO_2 exposure.

Tissue preparation and immunoblotting

The nucleus accumbens were rapidly dissected on ice, and homogenized using a sonicator in 100^{o}C 1% SDS with 1 mM NaF and 1 mM Na_3VO_4 as phosphatase inhibitors. Samples were boiled for 5 mins and stored at -80^{o}C. Protein concentrations of tissue samples were determined using the Lowry protocol (Lowry *et al*, 1951). A protein-antibody curve was constructed with control samples to determine optimal protein loading and antibody concentrations for immunoblotting. 30 μg protein of individual tissue samples were loaded onto 7.5% Tris-HCl Bio-Rad Ready-gels, separated by SDS-

polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes for immunoblotting. Nitrocellulose membranes were stained with 2.5% Ponceau S dye in 1% acetic acid/dH_2O to verify integrity of transferred protein. Membranes were washed in Tris buffered saline with Tween-20 (TTBS), and blocked with 5% non-fat dry milk in TTBS for 1hr at room temperature. Membranes were incubated with the following primary antibodies diluted in 5% non-fat dry milk in TTBS, 1:2000 for anti-phospho-GSK3α/β (Cell Signaling Technology, Beverly, MA, USA), and 1:5000 for anti-GSK3α/β (Santa Cruz Biotechnology, Santa Cruz, CA). The phospho-GSK3α/β antibody recognizes the phosphorylated form of GSK3α at serine 21(52 kDa) and also the form of GSK3β phosphorylated at serine 9 (47 kDa). Membranes were washed in TTBS and incubated with anti-rabbit or anti-mouse secondary antibodies conjugated to two different infra-red dyes (Li-cor Biosciences, Lincoln NE) at room temperature for 1h in a dark room. The infra- red dyes were detected as red and green by the Odyssey infrared imaging system (Li- cor). Secondary antibodies were diluted 1:10,000 in Odyssey blocking buffer with 0.2% Tween-20 (Li-cor Biosciences, Lincoln, NE). Membranes were visualized and proteins bands were quantified using the Odyssey infrared imaging system and software (Li- cor). Phosphorylated and total GSK3α/β were detected simultaneously in both red and green colors. To verify equal protein loading, membranes were stripped of antibodies using the New Blot nitro stripping buffer (Li-cor) and re-probed with anti-α-tubulin [1:80,000 (Sigma-Aldrich)]. Data are shown as ratios of densities of phosphorylated GSK3α/β to total GSK3α/β and total GSK3α/β to α-tubulin.

Data analysis

Data from immunoblot analyses were analyzed using GraphPad Prism by two-way ANOVA with factors of pretreatment (vehicle, SB 222200) and drug (saline, cocaine) followed by Bonferroni post hoc tests. Data for repeated studies analyses were analyzed using GraphPad Prism by two-way ANOVA with factors of pretreatment (vehicle, SB 222200) and drug (saline, cocaine) followed by Bonferroni post hoc tests. Statistical significance was determined at the alpha level of 0.05.

Results

Effect of the NK-3 receptor antagonist SB 222200 on GSK3α phosphorylation in the nucleus accumbens induced by cocaine

Phosphorylated GSK3α (Ser-21) and total GSK3α protein were measured in nucleus accumbens tissue samples from mice pretreated with either vehicle or SB 222200 30 mins before an acute saline or cocaine injection. Representative immunoblots of both GSK3α and total GSK3α are shown in Figure 4.1a. Statistical analysis of ratios of phosphorylated GSK3α to total GSK3α showed a significant main effect of Drug ($F(1,45)= 7.61$, $p< 0.01$, Figure 4.1a). Bonferroni post hoc comparisons showed increased phosphorylated GSK3α to total GSK3α ratios 20 mins after an acute cocaine injection in the nucleus accumbens ($p<0.05$, vehicle-saline vs. vehicle-cocaine groups). Administration of SB 222200 by itself did not significantly alter ratios of phosphorylated GSK3α to total GSK3α ($p>0.05$ vehicle-saline vs. 5SB-saline) or cocaine-induced increase in phosphorylated GSK3α to total GSK3α ratios ($p<0.05$ vehicle-saline vs. 5SB-cocaine). Ratios of total GSK3α to tubulin were unaltered by any drug treatment (Figure 4.1b). These data suggest that cocaine acutely increased phosphorylation of GSK3α at

serine 21 in the nucleus accumbens 20 mins post injection, however administration of SB 222200 had no effect on changes in GSK3α phosphorylation induced by acute cocaine.

Effect of pretreatment with the NK-3 receptor antagonist SB 222200 on cocaine-induced GSK3β phosphorylation in the nucleus accumbens

The nucleus accumbens from mice pretreated with either vehicle or SB 222200 for 30 mins before a saline or cocaine injection were examined for changes in levels of phosphorylated GSK3β (Ser-9) and total GSK3β protein. Representative immunoblots of both GSK3β and total GSK3β are shown in Figure 4.2a. Statistical analysis of ratios of phosphorylated GSK3β to total GSK3β showed significant differences between treatment groups ($F(1,45)= 7.1$, $p< 0.05$, Figure 4.2a). Post hoc comparisons revealed increased phosphorylated GSK3β to total GSK3β ratios 20 mins after an acute cocaine injection in the nucleus accumbens ($p<0.05$, vehicle-saline vs. vehicle-cocaine groups). There was also increased phosphorylated GSK3β to total GSK3β ratios after administration of SB 222200 with acute cocaine ($p<0.05$, vehicle- saline vs. SB-cocaine groups). Administration of SB 222200 by itself did not significantly alter ratios of phosphorylated GSK3β to total GSK3β ($p>0.05$ vehicle-saline vs. SB-saline). Ratios of total GSK3β protein to α-tubulin were unaltered by drug treatment (Figure 4.2b). These data suggest that an acute injection of cocaine increased phosphorylation of GSK3β at serine 9 in the nucleus accumbens 20 mins later, however SB 222200 administration had no effect on GSK3β phosphorylation after acute cocaine.

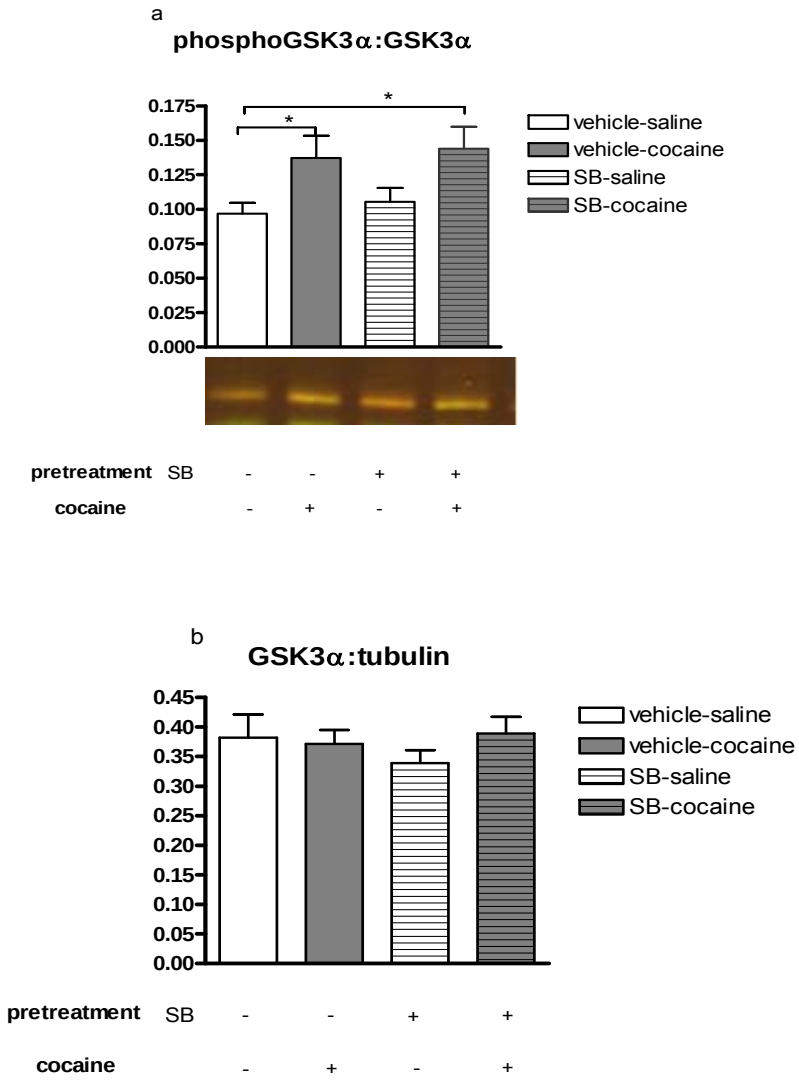

Figure 4.1. GSK3α phosphorylation in the nucleus accumbens after acute cocaine administration. Adult male CD-1 mice were injected with either vehicle or the NK-3 receptor antagonist SB 222200 (5 mg/kg s.c.) 30 mins prior to a cocaine (20 mg/kg, i.p.) injection. The nucleus accumbens was examined for changes in GSK3α phosphorylation 20 mins after cocaine administration. Representative immunoblots of both phosphorylated GSK3α (Ser-21) and total GSK3α of tissues from the nucleus accumbens of each treatment groups are also shown. Data are presented as mean ± SEM; N=12-13/group (* $p<0.05$).

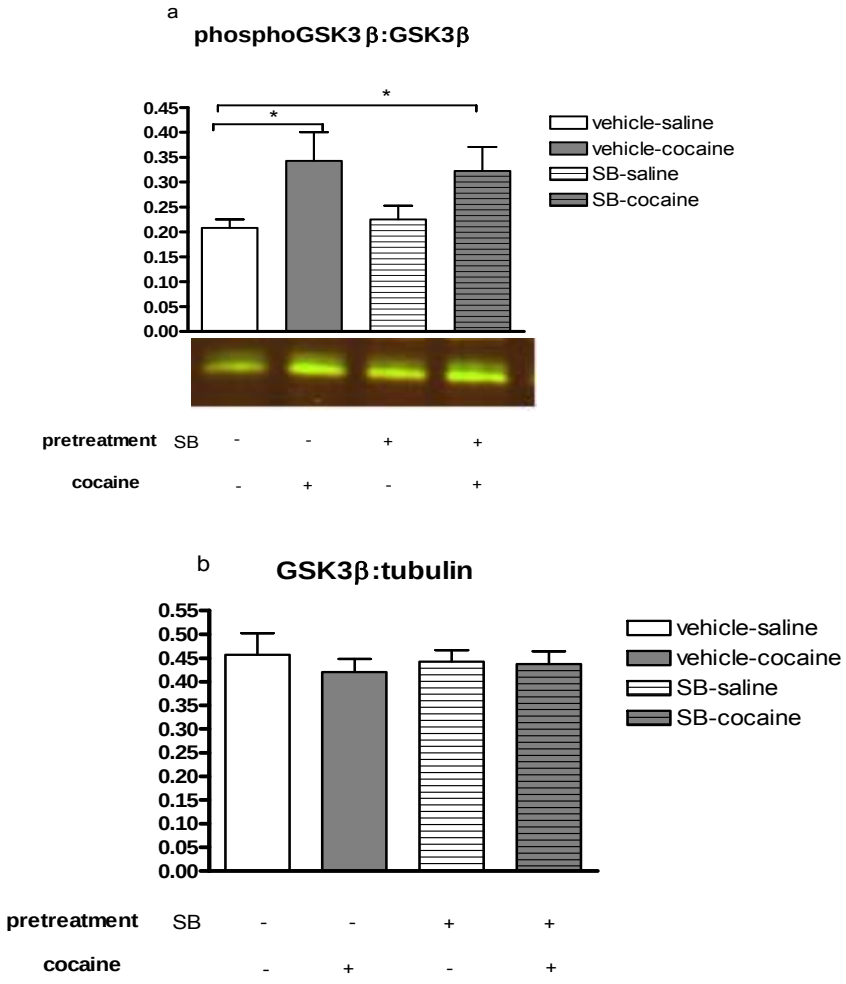

Figure 4.2. GSK3β phosphorylation in the nucleus accumbens after an acute injection of cocaine. Adult male CD-1 mice were injected with either vehicle or the NK-3 receptor antagonist SB 222200 (5 mg/kg s.c.) 30 mins prior to a cocaine (20 mg/kg, i.p.) injection. The nucleus accumbens was examined for changes in GSK3β phosphorylation 20 mins after cocaine administration. Representative immunoblots of both phosphorylated GSK3β (Ser-9) and total GSK3β of tissues from the nucleus accumbens of each treatment groups are also shown are also shown. Data are presented as mean ± SEM; N=12-13/group (* $p<0.05$).

Repeated cocaine and pretreatment with the NK-3 receptor antagonist SB 222200 alters GSK3α phosphorylation in the nucleus accumbens

Phosphorylated GSK3α (Ser-21) and total GSK3α protein were determined in nucleus accumbens tissue samples from mice pretreated with either vehicle or SB 222200 and saline or cocaine from 5 days, challenged with either saline or cocaine on day 13, and euthanized 20 mins later. Representative immunoblots of both GSK3α and total GSK3α are shown in Figure 4.3a. Statistical analysis of ratios of phosphorylated GSK3α to total GSK3α showed significant differences between treatment groups ($F(7,61)= 3.67$, $p< 0.01$, Figure 4.3a). Bonferroni post hoc comparisons showed increased phosphorylated GSK3α to total GSK3α ratios 20 mins after an acute cocaine injection in the nucleus accumbens ($p<0.05$, vehicle-saline-saline vs. vehicle-saline-cocaine groups). Repeated cocaine administration did not alter phosphorylated GSK3α to total GSK3α ratios after a subsequent cocaine challenge ($p>0.05$, vehicle-cocaine-saline vs. vehicle-cocaine-cocaine groups). There were increased phosphorylated GSK3α to total GSK3α ratios following a cocaine challenge after previous administration of SB 222200 with repeated cocaine ($p<0.05$, vehicle-cocaine-cocaine vs. SB-cocaine-cocaine groups). Repeated cocaine administration by itself did not significantly alter ratios of phosphorylated GSK3α to total GSK3α ($p>0.05$ vehicle-saline-saline vs. vehicle-cocaine-saline), nor did administration of SB 222200 by itself ($p>0.05$ vehicle-saline-saline vs. SB-saline-saline). Ratios of total GSK3α to tubulin were unaltered by any drug treatment (Figure 4.3b). These data show that cocaine acutely increased phosphorylation of GSK3α at serine 21 in the nucleus accumbens 20 mins post injection. GSK3α phosphorylation after a subsequent cocaine challenge was unchanged following repeated cocaine administration. SB 222200

administration prior to repeated cocaine reversed effects on GSK3α phosphorylation by repeated cocaine.

Repeated cocaine and pretreatment with the SB 222200 alters GSK3β phosphorylation in the nucleus accumbens

The nucleus accumbens from mice pretreated with either vehicle or SB 222200 and saline or cocaine and challenged with either saline or cocaine were examined for changes in levels of phosphorylated GSK3β (Ser-9) and total GSK3β protein. Representative immunoblots of both GSK3β and total GSK3β are shown in Figure 4.4a. Statistical analysis of ratios of phosphorylated GSK3β to total GSK3β showed significant differences between treatment groups ($F(7,58)= 3.7$, $p< 0.01$, Figure 4.4a). Post hoc comparisons revealed increased phosphorylated GSK3β to total GSK3β ratios 20 mins after an acute cocaine injection in the nucleus accumbens ($p<0.05$, vehicle-saline-saline vs. vehicle-saline-cocaine groups). Repeated cocaine produced no significant changes in ratios of phosphorylated GSK3β to total GSK3β after a subsequent cocaine challenge ($p>0.05$, vehicle-cocaine-saline vs. vehicle-cocaine-cocaine groups). However, there was increased phosphorylated GSK3β to total GSK3β ratios following a cocaine challenge after administration of SB 222200 with repeated cocaine ($p<0.05$, vehicle-cocaine-cocaine vs. SB-cocaine-cocaine groups). Repeated cocaine administration by itself did not significantly alter ratios of phosphorylated GSK3β to total GSK3β ($p>0.05$ vehicle-saline-saline vs. vehicle-cocaine-saline), nor did administration of SB 222200 ($p>0.05$ vehicle-saline-saline vs. SB-saline-saline). Ratios of total GSK3β protein to α-tubulin were unaltered by drug treatment (Figure 4.4b). These data demonstrate that an acute

injection of cocaine increased phosphorylation of GSK3β at serine 9 20 mins later in the nucleus accumbens, but repeated cocaine administration blunted changes in GSK3β phosphorylation after a subsequent cocaine challenge. In addition, SB 222200 administration prior to repeated cocaine reversed effects on GSK3β phosphorylation in the nucleus accumbens caused by repeated cocaine.

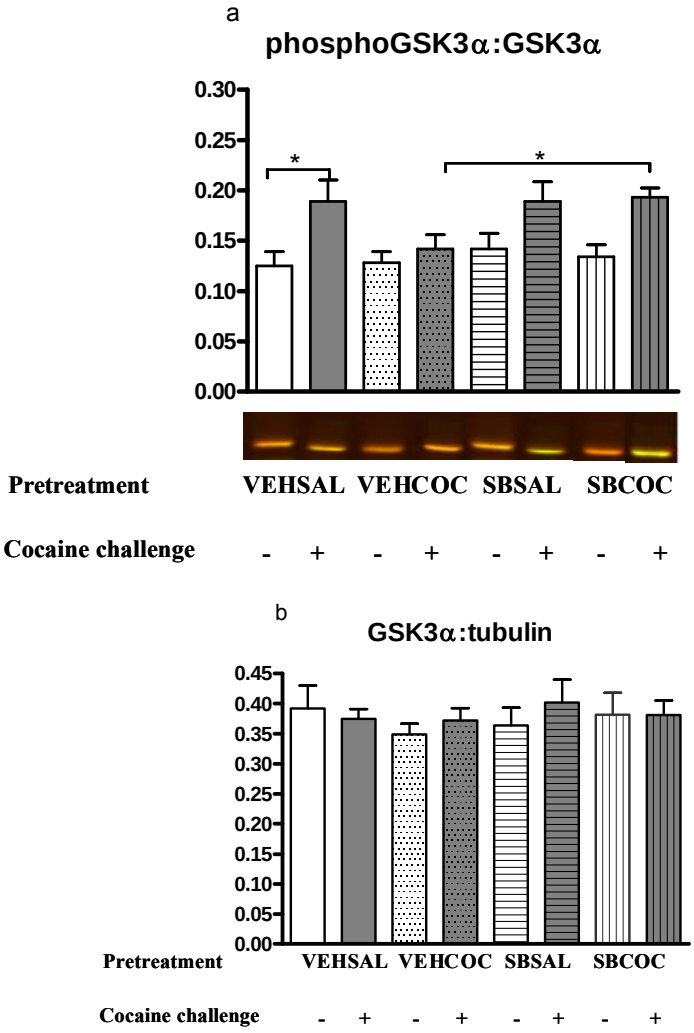

Figure 4.3. GSK3α phosphorylation in the nucleus accumbens after repeated cocaine administration and cocaine challenge. Adult male CD-1 mice were injected once daily with vehicle or SB and saline or cocaine (20 mg/kg) for 5 days, and 7 days later were challenged with either saline or cocaine. Nucleus accumbens were examined for changes in GSK3α phosphorylation 20 mins after the challenge. Representative immunoblots of both phosphorylated GSK3α (Ser-21) and total GSK3α of tissues from the nucleus accumbens of each treatment groups are shown. Data are presented as mean ± SEM; N=6-10/group (* $p<0.05$).

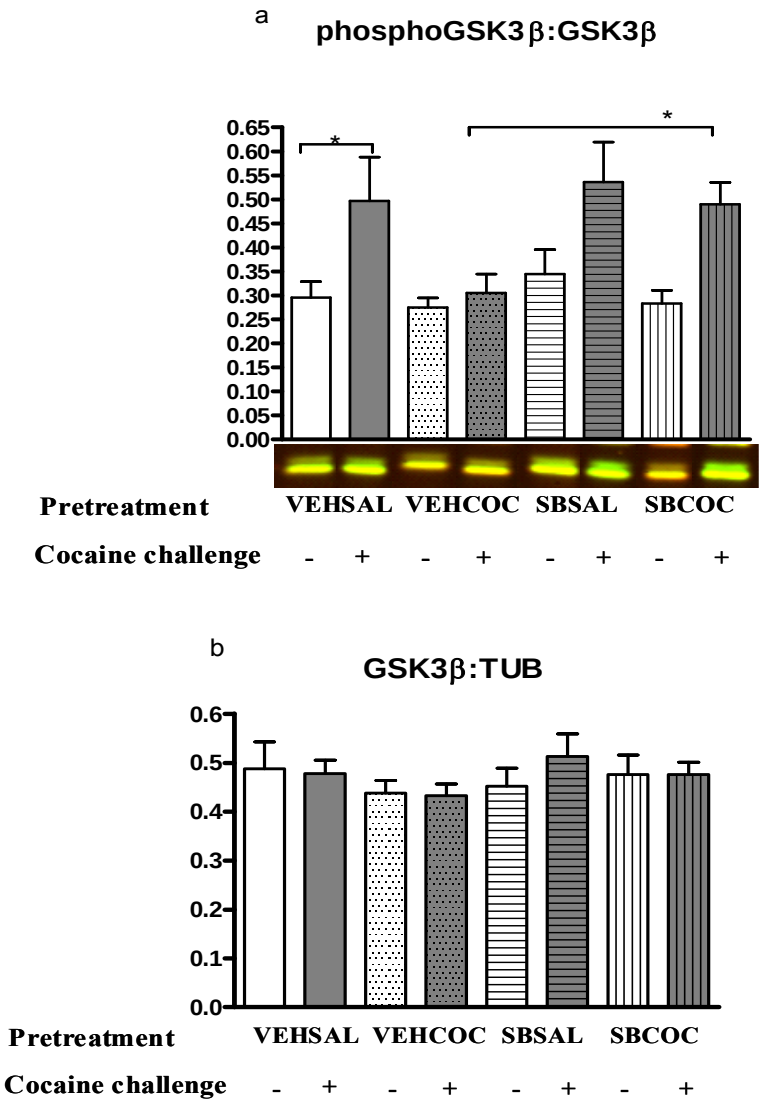

Figure 4.4. GSK3β phosphorylation in the nucleus accumbens after repeated cocaine administration and subsequent challenge. Adult male CD-1 mice were injected once daily with vehicle or SB and saline or cocaine (20 mg/kg) for 5 days. Seven days later, they were challenged with either saline or cocaine and examined for changes in GSK3 β phosphorylation 20 mins later. Representative immunoblots of both phosphorylated GSK3β (Ser-9) and total GSK3β protein of tissues from the nucleus accumbens of each treatment groups are shown. Data are presented as mean ± SEM; N=6-10/group (* $p<0.05$).

Discussion

Activity of the alpha and beta isoforms of GSK3 is regulated by phosphorylation at serine 21 and serine 9 residues, respectively, which leads to kinase inhibition (Alessi *et al*, 1996; Li *et al*, 2000; Svenningsson *et al*, 2003). GSK3 has been shown to phosphorylate transcription factors including cAMP responsive element binding protein (CREB) (Fiol *et al*, 1994), cmyc (Plyte *et al*, 1992), cjun (Boyle *et al*, 1991), and beta catenin (Seeling *et al*, 1999). Upon activation, GSK3 functions as a negative regulator of transcription factor activity (Jope *et al*, 2002). Conversely, inhibition of GSK3 activity through phosphorylation would lead to induction of transcription factor activity. In the present study, acute cocaine administration resulted in phosphorylation of GSK3 in the nucleus accumbens, an event that does not involve NK-3 receptors but may be mediated by dopamine receptor activation (Figure 4.5). Indirect activation of dopamine receptors in the striatum by psychostimulants such as amphetamine and cocaine have previously been shown to induce phosphorylation of GSK3 (Svenningsson *et al*, 2003). Previous studies have shown that acute cocaine induces activity of the transcription factor CREB that is involved in drug-induced neuroplastic events (McClung and Nestler, 2003). Decreases in GSK3β activity via phosphorylation causes an increase in CREB binding to DNA (Grimes *et al*, 2001a). Collectively, we propose that the observed cocaine-induced phosphorylation of GSK3 is mediated by dopamine receptors. GSK3 phosphorylation could eventually lead to activation of transcription factors such as CREB, which are involved in cocaine-induced neuroplasticity (McClung *et al*, 2003), (Figure 4.5).

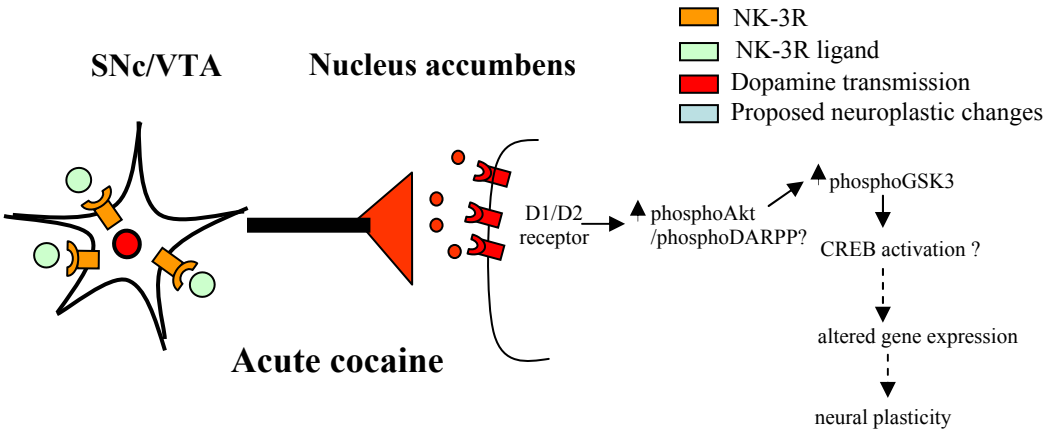

Figure 4.5. A proposed mechanism of acute cocaine-induced changes in GSK3 phosphorylation. Acute cocaine administration transiently induces phosphorylation of GSK3 in the nucleus accumbens through D1/D2 receptors, an effect that does not require activation of NK-3 receptors. GSK3 phosphorylation eventually can lead to neural plasticity by regulation of gene transcription through modulating CREB activity.

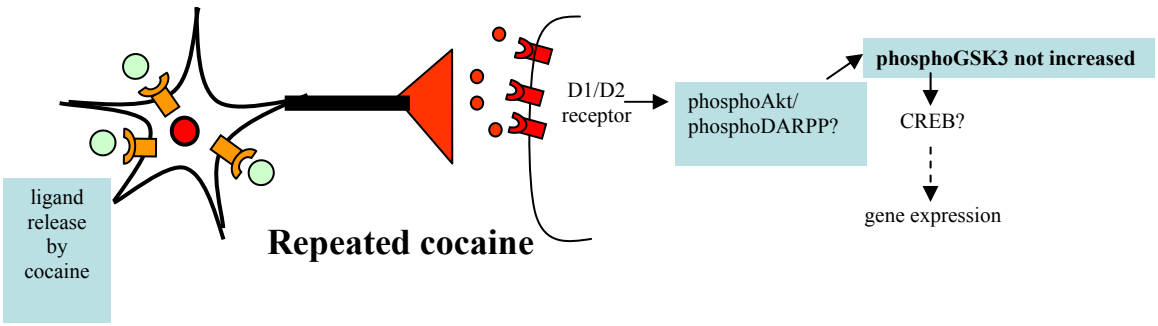

Figure 4.6. A proposed mechanism of repeated cocaine-induced changes in GSK3 phosphorylation. Repeated cocaine administration induces a state of impaired regulation of GSK3 activity in the nucleus accumbens, an effect that involves NK-3 receptor activation. The altered GSK3 activity is the cause of neuroplastic changes initially induced by acute cocaine administration.

Repeated intermittent administration of cocaine induces long-term molecular neuroadaptations in the nucleus accumbens (Anderson *et al*, 2005; Pierce *et al*, 1997; Vanderschuren *et al*, 2000). Some of these molecular adaptations have been previously described that involve activation and accumulation of the transcription factors CREB and variants of delta fos-B protein (Hope, 1994; McClung, Nestler, 2003). In the present study, we have demonstrated that repeated cocaine induces a state in which the increase in GSK3 phosphorylation in the nucleus accumbens after a subsequent cocaine challenge is absent (Figure 4.5). We propose that this state may result in impaired function of GSK3 as a negative regulator of transcription and gene expression. In addition, NK-3 receptor blockade reversed these changes in GSK3 phosphorylation induced by repeated cocaine, which implicates involvement of NK-3 receptor activity in this event (Figure 4.5). Acute as well as repeated cocaine administration increases preprotachykinin mRNA in the striatum (Adams *et al*, 2001; Mathieu-Kia *et al*, 1998). Therefore, activation of NK-3 receptors mediates long-term molecular changes in GSK3 activity in the nucleus accumbens following repeated cocaine administration.

Of particular interest, impaired control of GSK3 activity in the brain has been conceptualized to play an important role in mood disorders such as depression and bipolar disorder (Jope and Roh, 2006; Klein and Melton, 1996; Phiel and Klein, 2001). It is hypothesized that GSK3β may not be adequately controlled and may be aberrantly overactive in these conditions (Jope *et al*, 2006). This was initially based on the discovery that lithium, which is used to treat mood disorders, was a direct inhibitor of GSK3β (Jope *et al*, 2006; Klein *et al*, 1996; Munoz-Montano *et al*, 1997). In addition, selective serotonin reuptake inhibitors and tricyclic antidepressants such as fluoxetine and

imipiramine used in treatment of mood disorders have been shown to inhibit GSK3β activity via phosphorylation *in vivo* (Li *et al*, 2004). Conversely, selective GSK3 inhibitors have been shown to have anti-depressant like behavioral effects (Gould *et al*, 2004; Kaidanovich-Beilin *et al*, 2004). Our study shows similar impairment in GSK3α and β activity as a result of repeated cocaine administration that may play a role in behavioral sensitization. Therefore, in addition to affective disorders, we propose that the loss of inhibitory control of GSK3 occurs in behavioral sensitization, and may also involve changes in NK-3 receptor function.

Investigations on changes in GSK3 phosphorylation in the striatum induced by acute administration of psychostimulants are ongoing. Increases in phosphorylation of GSK3β after acute amphetamine and cocaine in the striatum have been reported (Svenningsson *et al*, 2003), but so has decreases in GSK3β phosphorylation (Beaulieu *et al*, 2004; Miller *et al*, 2009). The discrepancies in these reports appear to be due to a number of factors that include the timing of the measurement of changes in GSK3 phosphorylation after drug exposure, the brain regions involved, and the GSK3 isoforms. While Beaulieu et. al 2004 and Miller et. al 2009 demonstrate decreases in GSK3 between 30 mins to 90 mins after amphetamine and cocaine administration respectively, Miller and colleagues shows changes in GSK3β occurring in the caudate putamen and Beaulieu report changes in both GSK3α and β in the whole striatum. Svenningson et. al. 2003 demonstrates increases in GSK3β phosphorylation earlier within 15 mins of amphetamine and cocaine administration in the whole striatum. In our study, increases in GSK3α and β phosphorylation in the nucleus accumbens after acute cocaine administration 20 mins post administration, which seems to be in agreement with

Svenningson and colleagues. Unlike, Svennningson, our study demonstrated changes in the nucleus accumbens, and also shows novel effects of repeated cocaine administration in the apparent early induction of GSK3 phosphorylation, effects that are mediated by NK-3 receptor activity.

We postulate that the increase in GSK3 phosphorylation and inhibition of GSK3 activity 20 mins after an acute cocaine administration may have relevance in the drug-induced regulation of transcription factor activity and subsequent altered gene expression occurring after repeated cocaine administration. The significance of cocaine-induced changes in GSK3 phosphorylation in a functional context of acute behavioral hyperactivity remains a mystery. Studies have shown that inhibition of GSK3 activity by pharmacological compounds attenuates dopaminergic and cocaine-induced behaviors (Beaulieu *et al*, 2004; Miller *et al*, 2009) which may not be in agreement with our present findings of a transient increase in GSK3 phosphorylation and inhibition induced by acute cocaine that is not mediated by NK-3 receptors. We thereby propose that dynamic changes in GSK3 phosphorylation induced by acute cocaine may mediate behavioral hyperactivity. In order to resolve this, future investigation is warranted to closely examine the time-dependent changes in GSK3 phosphorylation by acute cocaine.

Our findings suggest that there are alterations in drug-induced increases in GSK3 phosphorylation and inhibition of kinase activity after repeated cocaine in the nucleus accumbens. These changes resulting from repeated cocaine also coincide with the manifestation of behavioral sensitization after a cocaine challenge, suggesting that the lack of increase in GSK3 phosphorylation and thus impaired inhibition of GSK3 activity in the nucleus accumbens by cocaine may be involved in the manifestation of sensitized

behavioral responses. The lack of increase in GSK3 phosphorylation as a result of repeated cocaine also involves NK-3 receptors since NK-3 receptor blockade with repeated cocaine resulted in an increase in GSK3 phosphorylation after a subsequent challenge. In addition, studies presented in the previous chapter demonstrated that NK-3 receptor activity modulated behavioral sensitized responses to cocaine. Therefore, we propose that NK-3 receptors are involved in alterations in GSK3 phosphorylation in the nucleus accumbens induced by cocaine that may underlie behavioral sensitization.

In summary, the present study demonstrates that cocaine acutely increases GSK3 phosphorylation in the nucleus accumbens, an event that does not involve NK-3 receptor activity. The induction of GSK3 phosphorylation is absent after repeated cocaine administration and subsequent cocaine challenge, and this molecular alteration in the nucleus accumbens involves NK-3 receptor activation. These findings point to neuroplastic changes in GSK3 phosphorylation in the nucleus accumbens induced by repeated cocaine that may mediate cocaine behavioral sensitization, events that require activation of NK-3 receptors.

CHAPTER 5

GENERAL DISCUSSION

Anatomical studies provide evidence in support of tachykinin-3 (NK-3) receptor expression in brain regions pertinent to behavioral hyperactivity and neuronal plasticity induced by cocaine and one such region is the striatum (Langlois *et al*, 2001; Mileusnic *et al*, 1999; Saffroy *et al*, 1988). In the striatum as well as in afferent regions such as the substantia nigra and ventral tegmental area, NK-3 receptor activity has been shown to modulate dopaminergic neurotransmission and behavioral hyperactivity (Bannon *et al*, 1995; Bishop and Walker, 2004; Keegan *et al*, 1992). In particular, cocaine-induced behaviors are altered by NK-3 receptor agonists and antagonists (Jocham *et al*, 2007; Jocham *et al*, 2006; Silva *et al*, 2008). GSK3β is a negative regulator of gene expression and transcription that has been shown to modulate dopamine- and cocaine-induced behavioral hyperactivity and sensitization (Beaulieu *et al*, 2004; Miller *et al*, 2009). This dissertation examined the role of NK-3 receptors in long-term changes in dopaminergic hyperactivity. In addition, NK-3 receptor involvement in cocaine-induced neuroplasticity as related to changes in GSK3 activity in the nucleus accumbens was also addressed. In particular, acute and long-term effects of NK-3 receptor blockade on cocaine-induced hyperactivity and behavioral sensitization was examined, and molecular mechanisms involving dopamine D1 receptors and GSK3 phosphorylation in the striatum were explored.

In agreement with past studies, acute NK-3 receptor blockade using the NK-3 receptor antagonist SB222200 attenuated cocaine-induced behavioral hyperactivity, particularly stereotypic activity. In contrast, repeated NK-3 receptor blockade enhanced

subsequent cocaine-induced stereotypic activity, possibly resulting from changes in dopamine D1 receptor activity and/or dopamine D1 receptor expression in the striatum. These alterations are comparable to effects of chronic administration of dopamine receptor antagonists that lead to dopamine receptor super-sensitivity (Hess *et al*, 1986; Hess *et al*, 1988). Collectively, the enhancement of subsequent behavioral responses to both cocaine and the dopamine D1 receptor agonist SKF 82958 and the increase in dopamine D1 receptor density in the striatum are suggestive of dopamine receptor super-sensitivity due to repeated NK-3 receptor blockade.

NK-3 receptor blockade also prevented the development and expression of cocaine behavioral sensitization, a phenomenon possibly involving regulation of GSK3 activity. Acute cocaine administration causes an early transient increase in phosphorylation of both GSK3α and β in the nucleus accumbens, however this cocaine-induced increase in GSK3 phosphorylation is absent after previous repeated cocaine exposure. These changes also coincide with the manifestation of behavioral sensitization to cocaine, suggesting that the absence of early GSK3 phosphorylation in the nucleus accumbens may be involved in the induction of sensitized behavioral responses. Moreover, NK-3 receptor blockade during repeated cocaine administration prevents cocaine-induced alterations in GSK3 phosphorylation, which indicates that NK-3 receptors are involved in changes in GSK3 phosphorylation in the nucleus accumbens induced by cocaine that may play a role in behavioral sensitization.

Studies outlined in this dissertation have led us to develop the following working hypotheses. Together with findings presented in the preceding chapters and from previous studies demonstrating that cocaine-induced behavioral responses are altered

after acute administration of NK-3 receptor ligands (Jocham *et al*, 2007; Jocham *et al*, 2006; Placenza *et al*, 2004; Silva *et al*, 2008), we postulate that NK-3 receptor activity modulates acute as well as long-term behavioral effects of cocaine. Studies have shown that administration of cocaine alters levels of preprotachykinin mRNA, which would correspond to increased levels of endogenous ligands of NK-3 receptors (Adams *et al*, 2001; Mathieu-Kia and Besson, 1998). Since functional studies indicate that acutely NK-3 receptors regulate dopaminergic neuronal activity and transmission to the striatum, we also postulate that NK-3 receptor activity modulates long-term dopaminergic activity presumably through altering dopaminergic neurotransmission. Lastly, we hypothesize that cocaine-induced neuroplasticity manifesting as sensitized behavioral responses may also concurrently involve alterations in GSK3 phosphorylation in the nucleus accumbens, a long-term-adaptation that is contingent upon NK-3 receptor activity. The resulting change in GSK3 phosphorylation could alter the ability of GSK3 to regulate gene expression and thereby modulate behavioral responses to cocaine. In support of this hypothesis, inhibition of GSK3 activity has been shown to alter cocaine-induced behavioral sensitized responses (Miller *et al*, 2009).

Future directions

Considering the postulations and supporting evidence presented above, some questions still remain to be addressed that pertain to involvement of NK-3 receptors in behavioral effects of cocaine. It will be of interest to determine whether other behavioral effects such as cocaine reward, cocaine-seeking behaviors and reinstatement to drug seeking behaviors are also modulated by NK-3 receptor activity. While there is some

evidence indicating NK-3 receptors are involved in the reinstatement of cocaine seeking behaviors (Placenza *et al*, 2004) it remains unclear the extent of NK-3 receptor involvement in cocaine reward (Jocham *et al*, 2007). Also, since cocaine-induced behavioral hyperactivity is presumed to be dependent on NK-3 receptor activation, investigations examining cocaine-induced activation of NK-3 receptors and initiation of Ca^{2+}dependent downstream signaling cascades may be warranted. The nature and/or extent of the long-term changes in dopaminergic neurotransmission and behaviors caused by prior administration of NK-3 receptor antagonists also needs further inquiry. In addition, while we have demonstrated changes in dopamine D1 receptor density in the striatum that accompany the change in behavioral response, it is also likely that there may be a compensatory increase in NK-3 receptors. The up-regulation of NK-3 receptors in the substantia nigra and/or VTA by prior administration of NK-3 receptor antagonists can also lead to enhanced dopaminergic transmission, and behaviors. These hypotheses can be investigated in studies that examine the onset of changes in dopamine-mediated behavior, changes in dopamine receptor expression, alterations in dopaminergic transmission to the striatum, and change in NK-3 receptors after prior NK-3 receptor antagonist administration.

NK-3 receptor activity is proposed to have a role in changes in GSK3 phosphorylation in the nucleus accumbens induced by repeated cocaine. GSK3β modulates activity of transcription factors implicated in cocaine-induced neuroplasticity such as CREB and delta fos B (Grimes *et al*, 2001a, b). However, possible involvement of NK-3 receptors in regulation of CREB and/or delta fos B after chronic cocaine administration remains largely unknown. Therefore, studies examining changes in CREB

and delta fos B in the nucleus accumbens after chronic cocaine and NK-3 receptor agonist administration are also warranted. We propose that studies addressing these issues may offer more insight into possible mechanisms of NK-3 receptor activity on behavioral effects of cocaine.

The findings in this dissertation add to growing evidence in support of functional role of NK-3 receptors in behavioral effects of cocaine that may have relevance in clinical aspects of cocaine abuse and dependence. A study by Foroud et. al. 2008 report significant associations of several SNPs in the TACR3 gene that encodes the NK-3 receptor with phenotypes of cocaine and alcohol co-abuse (Foroud *et al*, 2008). This finding may perhaps be useful in identifying subpopulations that may have differential responses to pharmacotherapies, or in identifying cocaine abusers who are at higher risk for severe drug dependence. NK-3 receptors have also been shown to have a role in mood and affective disorders such as anxiety and depression and have been proposed as a therapeutic target for treatment of these disorders (Panocka *et al*, 2001; Ribeiro *et al*, 1998). Furthermore, depression is a common symptom occurring during withdrawal from chronic cocaine use, and the presence of co-morbid mood disorders affects treatment prognosis in cocaine abusers (Poling *et al*, 2007). Therefore, NK-3 receptors may be a novel target for treatment of co-morbid mood disorders with cocaine abuse and also for the treatment of depression observed during cocaine withdrawal.

NK-3 receptors may also be involved in cocaine-induced toxic insult to the cardiovascular system. Acute and chronic cocaine effects on the cardiovascular system include myocardial infarction, acceleration of hypertension, coronary atherosclerosis, left ventricular hypertrophy, myocarditis and endocarditis that all can result in congestive

heart failure (Pozner *et al*, 2005). NK-3 receptors localized in the VTA have been shown to be involved in the autonomic regulation of cardiac function, in studies demonstrating that central administration of NK-3 receptor agonists and administration into the VTA increases systemic blood pressure and heart rate (Cellier *et al*, 1997; Deschamps *et al*, 2005; Picard *et al*, 1994; Roccon *et al*, 1996). Therefore, it is possible that NK-3 receptors may also have a functional role in cardiovascular effects of cocaine toxicity.

NK-3 receptors are being studied as potential therapeutic targets for treatment of various CNS disorders such as schizophrenia and Parkinson's Disease (Panocka *et al*, 2001; Ribeiro *et al*, 1998; Spooren *et al*, 2005) using various animal models. However, species differences in NK-3 receptor structure, expression in the central nervous system, and ligand selectivity (Buell *et al*, 1992; Langlois *et al*, 2001; Maggi, 1995; Mileusnic *et al*, 1999) have presented obstacles in the study of NK-3 receptors as a therapeutic target. From findings presented in this dissertation, additional obstacles also include possible consequences of chronic use of NK-3 receptor antagonists, causing dopamine receptor super-sensitivity, similar to what is observed with use of classical antipsychotics that cause tardive syndromes. Therefore, while targeting of NK-3 receptors may have some therapeutic potential, further preclinical studies are needed to better characterize NK-3 receptors as a target for treatment of cocaine addiction.

Conclusion

This dissertation has demonstrated a role of NK-3 receptors in modulating acute as well long-term cocaine-induced behavioral hyperactivity, presumably through altering dopaminergic neurotransmission in the striatum. Acute blockade of NK-3 receptors attenuates cocaine-induced behaviors, but in contrast repeated blockade of NK-3

receptors enhances behaviors and increases dopamine receptor expression in the striatum. In addition, NK-3 receptor activity modulates long-term behavioral effects of cocaine. NK-3 receptor blockade prevents the development and expression of behavioral sensitization to cocaine induced by repeated administration. The sensitized behavioral responses may be associated with changes in GSK3 phosphorylation in the nucleus accumbens. Lastly, there is a potential role of NK-3 receptors in cocaine abuse and dependence and as a therapeutic target for treatment of cocaine addiction.

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